JVI Journals.ASM.org

Murine Antibody Responses to Cleaved Soluble HIV-1 Envelope Trimers Are Highly Restricted in Specificity

Joyce K. Hu,[a,b] Jordan C. Crampton,[a,b] Albert Cupo,[c] Thomas Ketas,[c] Marit J. van Gils,[d] Kwinten Sliepen,[d] Steven W. de Taeye,[d] Devin Sok,[b,f,g] Gabriel Ozorowski,[b,e] Isaiah Deresa,[a,b] Robyn Stanfield,[h] Andrew B. Ward,[b,e] Dennis R. Burton,[b,f,g] Per Johan Klasse,[c] Rogier W. Sanders,[c,d] John P. Moore,[c] Shane Crotty[a,b]

Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA[a]; Center for HIV-1/AIDS Vaccine Immunology and Immunogen Discovery (CHAVI-ID), La Jolla, California, USA[b]; Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York, USA[c]; Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands[d]; Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, California, USA[e]; Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA[f]; Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Boston, Massachusetts, USA[g]; Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA[h]; Department of Medicine, University of California, San Diego School of Medicine, La Jolla, California, USA[i]

ABSTRACT

Generating neutralizing antibodies (nAbs) is a major goal of many current HIV-1 vaccine efforts. To be of practical value, these nAbs must be both potent and cross-reactive in order to be capable of preventing the transmission of the highly diverse and generally neutralization resistant (Tier-2) HIV-1 strains that are in circulation. The HIV-1 envelope glycoprotein (Env) spike is the only target for nAbs. To explore whether Tier-2 nAbs can be induced by Env proteins, we immunized conventional mice with soluble BG505 SOSIP.664 trimers that mimic the native Env spike. Here, we report that it is extremely difficult for murine B cells to recognize the Env epitopes necessary for inducing Tier-2 nAbs. Thus, while trimer-immunized mice raised Env-binding IgG Abs and had high-quality T follicular helper (Tfh) cell and germinal center (GC) responses, they did not make BG505.T332N nAbs. Epitope mapping studies showed that Ab responses in mice were specific to areas near the base of the soluble trimer. These areas are not well shielded by glycans and likely are occluded on virions, which is consistent with the lack of BG505.T332N nAbs. These data inform immunogen design and suggest that it is useful to obscure nonneutralizing epitopes presented on the base of soluble Env trimers and that the glycan shield of well-formed HIV Env trimers is virtually impenetrable for murine B cell receptors (BCRs).

IMPORTANCE

Human HIV vaccine efficacy trials have not generated meaningful neutralizing antibodies to circulating HIV strains. One possible hindrance has been the lack of immunogens that properly mimic the native conformation of the HIV envelope trimer protein. Here, we tested the first generation of soluble, native-like envelope trimer immunogens in a conventional mouse model. We attempted to generate neutralizing antibodies to neutralization-resistant circulating HIV strains. Various vaccine strategies failed to induce neutralizing antibodies to a neutralization-resistant HIV strain. Further analysis revealed that mouse antibodies targeted areas near the bottom of the soluble envelope trimers. These areas are not easily accessible on the HIV virion due to occlusion by the viral membrane and may have resulted from an absence of glycan shielding. Our results suggest that obscuring the bottom of soluble envelope trimers is a useful strategy to reduce antibody responses to epitopes that are not useful for virus neutralization.

Vaccines are key components of the public health armamentarium. Almost all licensed viral vaccines work by inducing neutralizing antibody (nAb) responses. However, generating nAbs that protect against the myriad of circulating human immunodeficiency virus type 1 (HIV-1) strains is extremely challenging. The only nAb target on HIV-1 is the envelope glycoprotein (Env) complex, a trimer of gp120-gp41 subunits that tolerate extensive amino acid sequence variation. The protein surfaces of the trimer are extensively shielded by N-linked glycans that help HIV-1 evade the antibody response but that are, paradoxically, themselves targets for a rare subset of broadly neutralizing antibodies (bnAbs). Thus, various bnAbs discovered in HIV-1-infected individuals that can neutralize most circulating HIV-1 isolates of multiple subtypes serve as paradigms for the types of antibody an Env vaccine needs to induce (1–5). The passive transfer of moderate doses of bnAbs protects macaques against challenge with simian immunodeficiency virus (SIV)/HIV-1 (SHIV-1) hybrid viruses,

Received 26 June 2015 **Accepted** 28 July 2015

Accepted manuscript posted online 5 August 2015

Citation Hu JK, Crampton JC, Cupo A, Ketas T, van Gils MJ, Sliepen K, de Taeye SW, Sok D, Ozorowski G, Deresa I, Stanfield R, Ward AB, Burton DR, Klasse PJ, Sanders RW, Moore JP, Crotty S. 2015. Murine antibody responses to cleaved soluble HIV-1 envelope trimers are highly restricted in specificity. J Virol 89:10383–10398. doi:10.1128/JVI.01653-15.

Editor: G. Silvestri

Address correspondence to Shane Crotty, shane@lji.org.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.01653-15.

doi:10.1128/JVI.01653-15

providing guidance about what bnAb titers a vaccine should induce to accomplish protective immunity (6–9). Epitope mapping studies combined with knowledge of the trimer structure reveal at least five conserved sites on the trimer as bnAb targets. In broad terms, bnAbs need to either penetrate the few gaps in the glycan shield or recognize a component of the glycan shield itself. However, can bnAbs be induced by vaccination?

In HIV-1-infected individuals, bnAbs generally require several years to develop during a complex process of affinity maturation. Most bnAbs have undergone extensive somatic hypermutation (SHM), particularly in the variable heavy-chain region (4, 10, 11). Mutations in framework regions also are critical for neutralization breadth (11), as they can facilitate antigen recognition and antibody flexibility (12). Therefore, driving SHM appears to be advantageous for the evolution of bnAbs (13). SHM takes place in germinal centers (GCs), which are dynamic structures in lymphoid organs where B cells undergo affinity maturation (14). During affinity maturation, T follicular helper (Tfh) cells provide survival and proliferation signals to antigen-presenting GC B cells (15). The latter divide, undergo SHM, and are selected by Tfh cells for further rounds of division and SHM in an iterative process (16). As Tfh cells are critical for regulating the process of affinity maturation, they play a key role in bnAb generation. During chronic SIV infection of macaques, the accumulation of Tfh cells in lymph nodes correlated with the magnitude of GC and IgG responses (17). Furthermore, the frequency of memory Tfh cells in blood correlated with the ability of HIV-1-infected individuals to make bnAbs (18). Therefore, learning how to improve Tfh cell differentiation would be valuable to HIV-1 vaccine design strategies.

The neutralization sensitivity of HIV-1 strains varies greatly and can be quantified. Viruses classified as Tier-1 are the most sensitive, while Tier-2 and Tier-3 viruses are increasingly resistant (19). Most Tier-1 viruses have acquired their atypical sensitivity to nAbs as a result of passage through T cell lines *in vitro* (20), although a small subset of circulating HIV-1 strains do have this phenotype (19). In contrast, most transmitted variants are in Tier-2 and -3 (19, 21, 22), which means that a vaccine will need to induce antibodies able to overcome their resistance mechanisms (21, 22). Tier-1 viruses are considered to have Env trimers that are more open than those of their more resistant counterparts, thereby exposing additional nAb epitopes, particularly those associated with the gp120-V3 region and the CD4-induced co-receptor binding site (CD4i) (19, 23, 24). These regions tend to be occluded on the more closed Tier-2 Env trimers, such that Abs to, for example, V3 are ineffective against the corresponding viruses (19, 23). Because the epitopes for Tier-1 and Tier-2 nAbs generally differ, a Tier-1 nAb response to, for example, V3 cannot be converted to Tier-2 by an affinity maturation process. Consequently, generating Tier-1 nAbs is not a stepping stone toward generating Tier-2 nAbs.

Inducing a Tier-1 nAb response by immunizing with Env proteins is straightforward, but generating Tier-2 nAbs is a major challenge. In human vaccine efficacy trials of monomeric gp120 proteins, Tier-2 nAbs were almost never seen at meaningful titers (25, 26). The gp120 monomer immunogens represent a single subunit of the native Env trimer; hence, they cannot present many nAb epitopes in their proper structural context. In addition, Tier-1 nAb and nonneutralizing antibody (non-nAb) epitopes are highly immunogenic on gp120 monomers, but the resulting antibodies are ineffective against Tier-2 viruses. The question is whether a new generation of Env immunogens that mimic the native trimer can overcome some of these limitations. The BG505 SOSIP.664 protein is the first of this class of recombinant native-like trimers (27–29). The BG505 SOSIP.664 trimers have been extensively studied *in vitro* and shown to possess many of the antigenicity properties of the corresponding virus, including the presentation of multiple tertiary and/or quaternary structure-dependent bnAb epitopes while occluding most non-nAb epitopes (27–31). Their high-resolution structure also has been determined (27–29, 32). These new trimer immunogens need to be studied in animal models to gauge their potential for inducing Tier-2 nAbs. In rabbits and macaques, the BG505 SOSIP.664 trimers induce nAbs against the autologous Tier-2 BG505.T332N response, as well as a Tier-1 nAb response dominated by V3 Abs, but they do not induce heterologous Tier-2 nAbs (33). The autologous Tier-2 response represents progress and may be a first step in various vaccine strategies intended to generate neutralization breadth. Many of these approaches will require a full harnessing of the immune system and its ability to drive SHM in GCs (34, 35).

The mouse model offers major advantages for an exploration of the immunology underlying vaccine-induced immunity, because so many tools and genetically engineered strains are available. Its relatively low cost is an additional asset, because more variables can be explored than is feasible in macaques. Conversely, it is well understood in the Env vaccine field that mouse serum can interfere with HIV-1 neutralization assays *in vitro* (36), and there are also concerns about structural differences between mouse and human antibodies, such as the length of the CDR3 region.

Here, we have immunized mice with BG505 SOSIP.664 trimers under various conditions to assess whether they induce Tier-1 and/or autologous Tier-2 responses and to study various immunological parameters that may be important for the induction of bnAbs.

MATERIALS AND METHODS

Ethics statement. The mouse experiments were conducted in strict compliance with the La Jolla Institute for Allergy and Immunology Animal Care Committee (Office of Laboratory Animal Welfare assurance number A3779-01), who approved all animal care and protocol used (protocol license number AP006-SC1-0612). The mouse care and use protocol adheres to the Public Health Service (PHS) policy on the humane care and use of laboratory animals (Department of Health and Human Services) and the *Guide for the Care and Use of Laboratory Animals* (eighth edition) (37). The rabbit experiments were performed at Covance, Inc., and were approved by the Covance, Inc., IACUC (project license number 0014-15) in compliance with the U.S. Department of Agriculture's Animal Welfare Act (38), the *Guide for the Care and Use of Laboratory Animals* (37), and the National Institutes of Health, Office of Laboratory Animal Welfare.

Animals, immunizations, reagents, and surgeries for immunizations. BALB/cJ, 129S1/SvImJ, and C57BL/6J mice (Jackson Laboratory) or Crl:CFW(SW)/Swiss Webster mice (Charles River) were immunized at 6 to 10 weeks of age via the indicated routes (footpad, base of tail, interscapular, or intramuscular) and given subsequent booster immunizations. The following reagents were used in mouse vaccine strategies: adjuvants Iscomatrix (CSL Ltd.), Abisco-100 (Novavax), Addavax (InvivoGen), Adjuplex source, Complete Freund's adjuvant (InvivoGen), and Incomplete Freund's adjuvant (InvivoGen); Toll-like receptor (TLR) agonists MPLA-SM (10 μg/mouse; InvivoGen), R848 (20 μg/mouse; InvivoGen), and ODN 1826 (20 μg/mouse; InvivoGen); glycolipid analog 7DW8-5 (0.2 μg/mouse; Funakoshi Co. Ltd.); NK cell-depleting anti-Asialo-GM1 (39, 40) (20 μl/mouse intraperitoneally [i.p.] at day −1; BioLegend); anti-mouse CD178/Fas-L (100 μg/mouse i.p. at days 15, 17,

and 19 postimmunization and boost; eBioscience); anti-mouse CD30 (100 μg/mouse i.p. at days 15, 17, and 19 postimmunization and boost; eBioscience); recombinant human BAFF (10 μg/mouse i.p. each day between day −10 and day −1; PeproTech); recombinant mouse interleukin-6 (IL-6; 2 μg/mouse i.p. at the immunization; PeproTech); clodronate disodium (CLD; 4 mg/mouse; Sigma); and AKB-4924 (100 μg/mouse i.p.; Aerpio Therapeutics).

Seven-day (1007D or 2001; Alzet) or 14-day (1002 or 2002; Alzet) osmotic pumps were used for immunizations. For 7-day slow-release immunizations, smaller pumps (100-μl volume) were used for most experiments to deliver 50 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix , and larger pumps (200-μl volume) were used for a few experiments to deliver 50 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix. For 14-day immunizations, either two small pumps (100-μl volume each) or one large pump (200-μl volume) was used to deliver 100 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix. Larger volumes were required, because BG505 SOSIP.664 trimers were maintained at an approximately 0.5 mg/ml concentration to decrease the aggregation of trimers. In one experiment, Addavax was used in pumps in a 1:1 ratio with BG505 SOSIP.664 trimers (see Table S5 in the supplemental material). Pumps were implanted subcutaneously at the midscapular level into mice. Interscapular bolus immunization was given in pump-implanted mice as indicated.

New Zealand White rabbits (Covance Inc.) were immunized intramuscularly with 30 μg BG505 SOSIP.664 trimers in 75 U of Iscomatrix adjuvant.

Env trimers for immunizations. BG505 SOSIP.664 trimers were produced and purified as previously reported (27, 41). Briefly, the proteins were expressed in a human embryonic kidney 293 cell line expressing the simian virus 40 (SV40) large T-antigen (HEK293T) (Invitrogen) or Chinese hamster ovary (CHO) cells (Invitrogen) by transient transfection and purified using a 2G12 monoclonal antibody (MAb) affinity column followed by size-exclusion chromatography (SEC). YU2 gp140-F trimers were produced as previously described (42). The BG505 SOSIP.664 trimers were aliquoted at approximately 0.5 mg/ml and stored at −80°C. Once thawed, the BG505 SOSIP.664 trimers were used immediately or, in rare cases, kept at 4°C for up to 3 days for use.

Flow cytometry. C-terminus avi-tagged BG505.664 SOSIP trimers were produced as previously described (31) and biotinylated using the BirA-500 kit (Avidity) per the instructions. Biotinylated BG505 SOSIP.664 trimers were aliquoted and stored at −80°C. After thawing, biotinylated BG505.664 SOSIP trimers were tetramerized with streptavidin-AF647 (Life Technologies) and incubated with cells at 37°C for 1 h. Biotinylated BG505 SOSIP.664 trimer probes were used fresh within a day after thaw and tetramerization. Other stains were performed as previously described (43).

ELISAs. For BG505 gp140 IgG-specific enzyme-linked immunosorbent assays (ELISAs), 96-well MaxiSorp plates (Thermo Scientific) were coated overnight at 4°C with Ab D7324 (from Aalto Bio Reagents Ltd.) at 5 μg/ml in 0.1 M $NaHCO_3$, pH 9.5 (50 μl/well). After washing with PBS–0.5% Tween 20, plates were blocked with 2% skim milk for 1.5 h at room temperature (RT). After washing, 0.3 μg/ml D7324-tagged BG505 SOSIP.664 trimers were added in PBS–2% milk–10% sheep serum for 1 h at RT. C-terminus D7324-tagged BG505 SOSIP.664 trimers were made as previously described (27). After washing, mouse serum was added in a dilution series in PBS–2% milk–10% sheep serum for 1.5 h at RT. After washing, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and fragment-specific Fcγ (Jackson ImmunoResearch) was added at 1:2,000 in PBS–2% milk–10% sheep serum for 1 h at RT. Colorimetric detection was performed using a TMB substrate kit (Thermo Scientific). Color development was stopped after approximately 15 min with 2N H_2SO_4, and absorption was measured at 450 nm. For V3-Fc competition ELISAs, mouse serum samples were incubated with 4 μg V3-Fc fusion protein overnight (12 to 18 h). Each mouse serum sample incubated with or without V3-Fc was plated in a dilution series on the same ELISA plate. Total mouse IgG ELISAs were performed with the following changes: donkey anti-mouse IgG (H+L) was used to coat MaxiSorp plates, and mouse IgG (BioXCell) was used as a standard.

Generation of V3-Fc fusion protein. The V3-Fc fusion construct contained a CD5 antigen leader, followed by residues 291 to 336 from HIV-1 BG505 gp120 and then residues 224 to 478 of a human IgG Fc domain, including the hinge, CH2, and CH3 regions, similar to a construct previously described (44). These residues were cloned into the pHCMV3 vector for mammalian expression. The BG505 V3-Fc fusion protein was transfected in 293F cells using 293Fectin (Life Technologies) by following the manufacturer's protocol. The supernatant was harvested 4 to 5 days post-transfection and purified over a protein A column. Following washing with phosphate-buffered saline, bound protein was eluted using 0.1 M citric acid (pH 3.0) and dialyzed against phosphate-buffered saline. V3-Fc was aliquoted and frozen at −80°C. Once thawed, V3-Fc was used immediately or kept at 4°C for up to 5 days for use. The stability of V3-Fc was confirmed for every V3-Fc competition ELISA. This was done by determining if V3-Fc blocked binding of the V3-specific MAb 14e to BG505 SOSIP.664 trimers compared to the bnAb VRC01 (a negative control).

Purification of mouse serum IgG. Mouse blood was collected and spun at maximum speed in a tabletop centrifuge to separate serum. Mouse serum samples were purified using a 0.2-ml protein G spin kit (product no. 89949; Thermo Scientific) per the instructions. Briefly, approximately 600 μl of serum was added to each column for purification, and three eluted fractions of mouse IgG were collected and combined. The low-pH elution buffer supplied in the kit was used for neutralization assays. High-salt elution buffers, including the gentle elution buffer (Thermo Scientific) and $MgCl_2$ elution buffers, caused nonspecific neutralization of Tier-1 and Tier-2 HIV and control vesicular stomatitis virus (VSV) pseudoviruses in neutralization assays. The concentrations of total mouse IgG from prepurification serum and the postpurification elution were assayed by ELISA (see "ELISAs" above) to determine the dilution of IgG from original serum.

Biosensor binding kinetics assays. Biosensor binding kinetics assays were performed on the Pall ForteBio Octet Red96 system. The concentration of purified mouse IgG from serum was measured using a total mouse IgG ELISA (described above) or a Pierce bicinichoninic acid (BCA) protein assay kit (Thermo Fisher). Octet kinetic buffer (PBS, pH 7.4, 0.1% BSA, 0.02% Tween 20, 0.01% NaN_3) was used in all steps (baseline, load, baseline, association, and dissociation, in sequential order). C-terminus avi-tagged BG505 SOSIP.664 trimers were biotinylated (see "Flow cytometry" above).

For binding kinetics of MAbs and bnAbs (obtained from the International AIDS Vaccine Initiative), 7 μg/ml or 1 μg/ml biotinylated BG505 SOSIP.664 trimers were loaded onto biosensors for 30 min or 1 h, respectively, and 20 μg/ml of MAb was associated for 30 min or 1 h and dissociated for 30 min or 1 h, respectively. For competition assays (baseline, load, baseline, association, and association, in sequential order), 1 μg/ml biotinylated BG505 SOSIP.664 trimers were loaded onto biosensors for 1 h. If human MAb was associated first, 20 μg/ml was associated for 1 h, followed by 20 μg/ml of mouse IgG plus 20 μg/ml of the associated MAb for 30 min. If mouse IgG was associated first, 20 μg/ml was associated for 3 h to saturate the binding of the mouse IgG. Afterwards, 20 μg/ml bnAb plus 20 μg/ml mouse IgG were associated for 30 min. Analysis was performed on the ForteBio data analysis software, version 7.1.

Neutralization assays. Tranzyme-β-galactosidase and luciferase (TZM-bl) reporter cell lines (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, John C. Kappes, Xiaoyun Wu, and Tranzyme Inc., Durham, NC) are Henrietta Lacks (HeLa) cell lines (cervical cancer cells) stably expressing high levels of CD4 and the coreceptors CCR5 and CXCR4, and they contain the β-galactosidase and luciferase genes under the control of the HIV-1 long terminal repeat promoter. TZM-bl neutralization assays were performed as previously described (27, 45, 46), with the following modifications. Pseudovirus TZM-bl cells were seeded at a concentration of 1×10^4 cells per well of

a 96-well plate. Sera were heat inactivated at 56°C for 1 h, and purified IgG was directly used without heat inactivation. Pseudovirus was incubated with sera or purified IgG at 37°C for 1 h before infection of TZM-bl cells. CD4-IgG2 was used as a positive control for neutralization. Although the BG505.T332N envelope is able to bind to soluble CD4 (23, 30), BG505.T332N pseudoviruses are relatively resistant to CD4-IgG2 neutralization and, like Tier-2 viruses and primary HIV-1 isolates, require higher concentrations of soluble CD4 or CD4-IgG than neutralization-sensitive Tier-1 viruses (47, 48).

Negative-stain EM. BG505 SOSIP.664 envelope proteins in Addavax or Iscomatrix or in pumps with Iscomatrix were prepared for negative-stain electron microscopy (EM) analysis as previously described (27, 49, 50). Briefly, a 3-μl aliquot containing 0.01 to 0.05 mg/ml of Env protein (as determined by UV A_{280} using the theoretical extinction coefficient based on peptide sequence alone) was applied for 5 s onto a carbon-coated 400 Cu mesh grid that had been glow discharged at 20 mA for 30 s and then negatively stained with 2% (wt/vol) uranyl formate for 60 s. Grids were screened to assess stain quality and particle distribution. The sample concentration was adjusted and grids remade until particle overlap on the grid surface was minimized. Data were collected on either an FEI Tecnai T12 electron microscope operating at 120 keV, with an electron dose of ~25 $e^-/Å^2$ and a magnification of 52,000× that resulted in a pixel size of 2.05 Å at the specimen plane, or an FEI Talos electron microscope operating at 200 keV, with an electron dose of ~25 $e^-/Å^2$ and a magnification of 73,000× that resulted in a pixel size of 1.98 Å at the specimen plane. Images were acquired with a Tietz TemCam-F416 CMOS camera (FEI Tecnai T12) or FEI Ceta 16M camera (FEI Talos) using a nominal defocus range of 1,000 to 1,500.

Data processing methods were adapted from those used previously (50). Two-dimensional (2D) class averages were labeled "closed," "open," or "nonnative" as described previously (50). The amount of native-like particles was defined as the sum of closed and open particles.

Statistical analysis. Prism 6 software was used to plot geometric means and geometric standard errors of the means for log-based graphs or means and standard errors of the means for linear-based graphs. Statistical analysis was performed using Mann-Whitney (two-tailed) *t* tests.

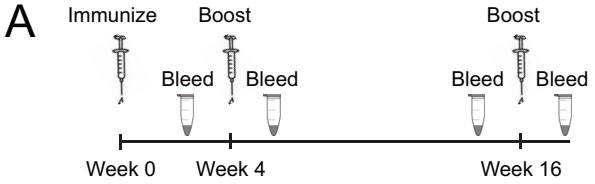
RESULTS

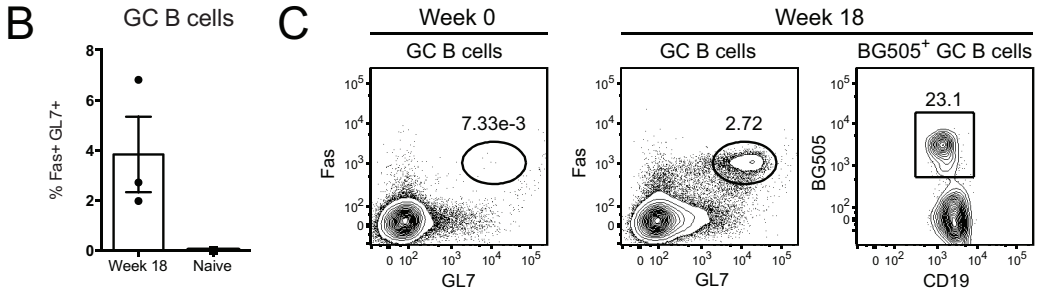
BG505 SOSIP.664 trimers are immunogenic and can elicit Tier-1 nAbs in mice. GCs are almost certainly central to the development of HIV-1 nAbs. We first compared GC responses in the commonly used mouse strains C57BL/6, BALB/c, and 129/Sv using the model antigen keyhole limpet hemocyanin (KLH). Tfh cells, GC Tfh cells, GC B cells, and plasma cells (PCs) in draining lymph nodes were quantified in the period 5 to 28 days postimmunization (see Fig. S1A to C in the supplemental material). BALB/c and 129/Sv mice had 2-fold higher frequencies of GC B cells at the peak of the B cell response than C57BL/6 mice (see Fig. S1A). GC responses also remained active substantially longer in BALB/c and 129/Sv mice. Similar results were obtained with an HIV Env immunogen tested in multiple mouse strains (data not shown). Accordingly, BALB/c and 129/Sv strains were used to study the BG505 SOSIP.664 trimer immunogens.

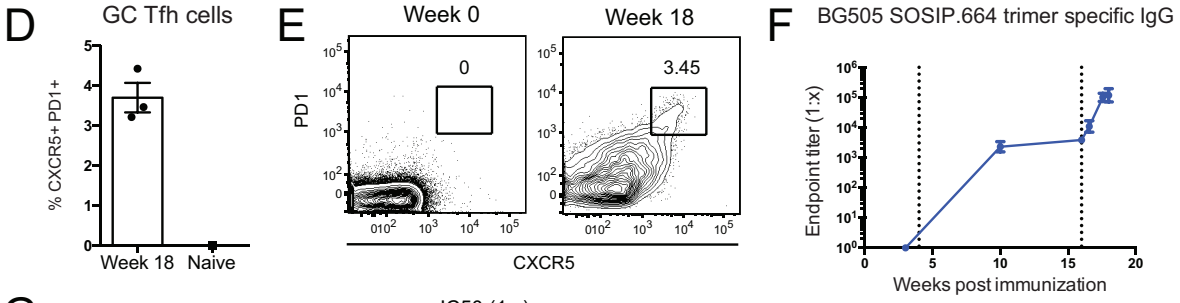
One conceivable challenge for BG505 SOSIP.664 trimer immunizations was that native-like compact Env trimers may be less immunogenic for inducing CD4 T cell and B cell responses than gp120 or poorly formed trimers. YU2 gp140-F proteins have been shown to have irregular shapes and open conformations that expose many nonneutralizing epitopes (33, 51). Thus, YU2 gp140-F was included as a general immunogenicity control. There were early GC Tfh cell and B cell responses in BALB/c mice after a single immunization with the Env trimers (see Fig. S2A and B in the supplemental material). Responses were of a magnitude comparable to those seen in mice immunized with YU2 gp140-F Env proteins (see Fig. S2A to F) (51). Thus, both native-like and nonnative Env proteins appear similarly immunogenic with respect to the magnitude of the CD4 T cell and B cell responses elicited.

Subsequent trimer immunizations were modeled on schedules developed for licensed human vaccines (52–54). Thus, the mice were given BG505 SOSIP.664 trimers three times. An initial immunization was followed by a 2nd immunization at 1 to 2 months, and the 3rd immunization was given after an extended rest period of 3 to 4 months (Fig. 1A). The primary endpoint readout was a neutralization assay to measure the induction of Tier-1 and Tier-2 nAbs. However, mouse serum contains unidentified components that display nonspecific antiviral activity (36, 55). These components also are found in other species (e.g., rabbits, macaques, and humans [36]) and interfere with neutralization assays, which measure the overall inhibition of HIV-1 replication in the presence of the added serum. We confirmed the existence of this problem by showing that sera from mice immunized with adjuvant alone caused nonspecific neutralization of both the HIV-1 BG505.T332N virus and vesicular stomatitis virus (VSV) when added at dilutions of <1:200 and >1:1600, respectively (see Fig. S3A in the supplemental material). This range of nonmeasurable neutralization titers (i.e., <1:200) is relevant because titers in this range (i.e., ~1:100) are associated with protection of macaques in passive transfer experiments (56). Therefore, we purified IgG from the mouse sera (see Materials and Methods) and showed that this procedure eliminated the nonspecific inhibitory factors. Thus, IgG purified from the sera of the adjuvant-alone immunized mice did not neutralize the BG505.T332N virus or VSV at dilutions as low as 1:7 (see Fig. S3B and C). In a confirmatory experiment, IgG purified from mice immunized with the irrelevant protein KLH also failed to neutralize the same two test viruses when added in amounts equivalent to serum dilutions as low as 1:9 (see Fig. S3C). Purified IgG samples from BG505 SOSIP.664 trimer-immunized animals then were tested for neutralization activity (see Fig. S4A and B). No Tier-1 or Tier-2 nAbs were detected after the final immunization (see Fig. S4B). However, we also noted that BG505 anti-trimer binding antibody IgG titers were low at this time point, and that the 3rd immunization had not boosted the antibody responses overall (see Fig. S4C).

We postulated that the 3rd immunization was ineffective, because trimer-specific Abs present had bound concurrently to the trimer immunogen and reduced trimer availability for direct binding by B cells (see Fig. S4C in the supplemental material), as has been observed in model antigen systems (57, 58). Thus, a shortage of available antigen may preclude a significant B cell recall response in draining lymph nodes. To test this hypothesis, we increased the dose of trimers from 10 to 20 μg in the 3rd immunization and observed an anamnestic response (Fig. 1). Robust GC responses were in draining lymph nodes 2 weeks after the 3rd immunization, as seen by the presence of GC B cells (Fig. 1B and C), BG505 Env-specific GC B cells (Fig. 1C), and GC Tfh cells (Fig. 1D and E). Moreover, trimer-specific IgG titers in the serum also were increased at this time (Fig. 1F), and Tier-1 nAbs now were detectable (Fig. 1G). Despite these positive responses to the 3rd immunization, we did not see the appearance of nAbs against the autologous Tier-2 BG505.T332N virus (Fig. 1G and H). A series of other immunization strategies yielded similar outcomes in that they were not able to induce autologous Tier-2 nAbs (Fig. 1I; also see Tables S1 to S6 in the supplemental material). While Tier-1



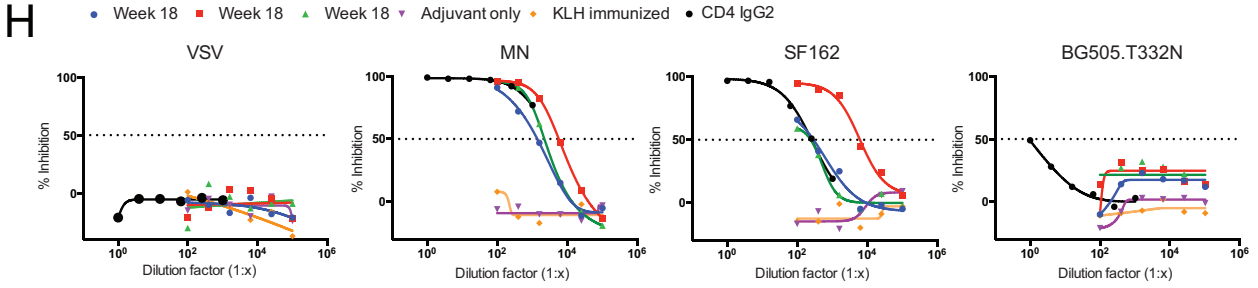


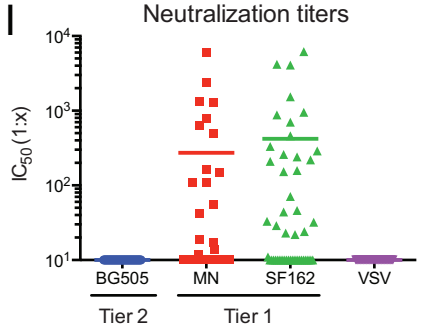


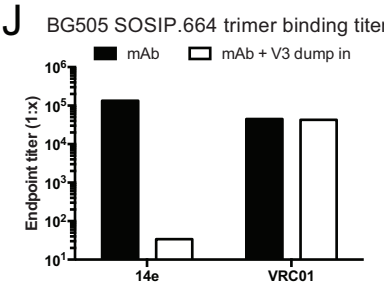
G

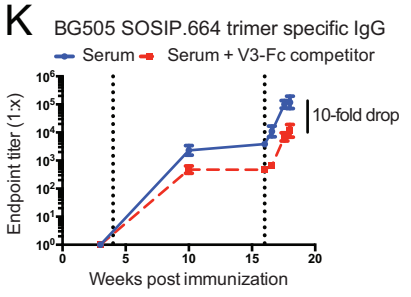
IC50 (1:x)

	Tier 2 Clade A	Tier 1 Clade B viruses		Control
	BG505.T332N	MN	SF162	VSV
Week 18	<100	1300	290	<100
Week 18	<100	6000	6200	<100
Week 18	<100	2400	240	<100
Adjuvant only	<100	<100	<100	<100
KLH immunized	<100	<100	<100	<100
CD4 IgG2 (μg/ml)	>50	<0.049	0.19	>50









nAbs often were induced, no single immunization strategy was notably better at Tier-1 nAb elicitation than the others (see Fig. S5 in the supplemental material).

To gain insight into why Tier-2 nAbs were not developing, we first compared results from BG505 SOSIP.664 trimer immunization mice against those from BG505 SOSIP.664-immunized rabbits. Mice made BG505 SOSIP trimer binding IgG ELISA titers in a range similar to that of rabbits, but the mouse IgG failed to neutralize autologous BG505 viruses (see Fig. S6A in the supplemental material). However, mice were capable of generating cross-reactive Tier-1 nAbs similar to those of rabbits (see Fig. S6B) and had gp120 IgG ELISA titers similar to those of rabbits (see Fig. S6C). The trimers indeed were capable of inducing a Tier-2 autologous nAb response, implying that mice were more restricted in their Ab responses to BG505 SOSIP trimers. We next sought to understand what limits the Tier-2 nAb response to the trimers in mice.

The V3 region of gp120 elicits an immunodominant Ab response in many Env vaccine and HIV-1 infection contexts (59, 60). Anti-V3 Abs and MAbs strongly neutralize Tier-1 viruses but usually have no meaningful impact on Tier-2 HIV-1 viruses, including BG505.T332N, because the V3 region is sequestered on the Env trimer prior to CD4 binding (19, 23). The structure of the BG505 SOSIP.664 trimer shows that V3 is poorly exposed, which is consistent with it not being a neutralization site on the corresponding virus (27–29, 61). However, trimers are not static entities but undergo conformational transitions over time (23, 62). Thus, the V3 region of the BG505 SOSIP.664 trimers can become exposed *in vitro* when the normally closed conformation opens. Therefore, we considered the possibility that a nonneutralizing, immunodominant Ab response to V3 is interfering with the generation of the Tier-2 autologous response in mice while at the same time contributing to the Tier-1 nAb response.

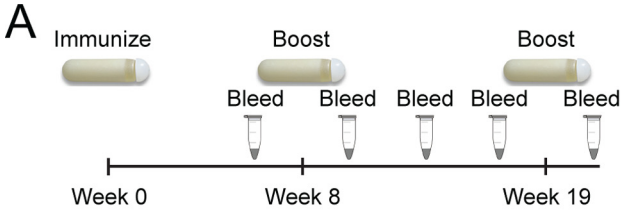
Under ELISA conditions, bnAbs bind to BG505 SOSIP.664 trimers, but so do V3 non-nAbs (27). Therefore, we used a V3 competition assay to assess the contribution of V3-specific Abs to the trimer-specific titers by ELISA. To do this, V3-Fc fusion proteins were incubated with the sera prior to addition to D7324-immobilized BG505 SOSIP.664-D7324 trimers, and the fold reduction in trimer binding was quantified. The competition ELISA was validated using the V3-specific MAb 14e and the CD4 binding site (CD4bs)-specific bnAb VRC01. This control experiment showed that 14e binding to the trimer was completely inhibited by prior exposure to the V3-Fc protein, leading to an ~5,000-fold titer reduction, but no such inhibition was seen with VRC01 (Fig. 1I). Hence, the V3-Fc fusion protein prevents the binding of a V3 MAb to the trimer but does so in an epitope-specific manner. We then used the same method to analyze mouse sera.

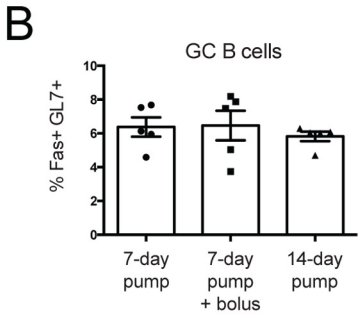
In the presence of the V3-Fc fusion protein competitor, the anti-trimer IgG ELISA titers decreased by 10-fold, raising the concern that a potentially large fraction of Abs was directed toward the V3 protein (Fig. 1J). Hence, one of the immunodominant responses to the BG505 SOSIP.664 trimers in mice involves the induction of Abs to V3 epitopes that are not associated with neutralization of Tier-2 viruses, including the autologous virus BG505.T332N.

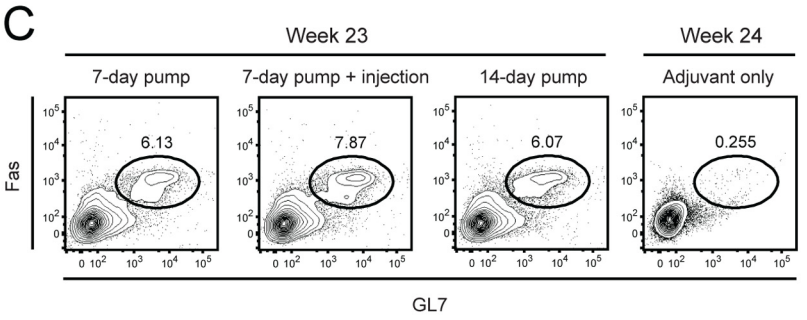
Antigen delivery via osmotic pumps reduces the immunodominant response to the V3 loop. The above-described experiments show that an immunodominant, V3-directed Ab response arises when BG505 SOSIP.664 trimers are delivered to mice by a bolus injection. We considered the possibility that a V3-specific B cell response distracts the immune response from targeting epitopes relevant for neutralizing the Tier-2 BG505.T332N virus. The exposure of the V3 region of BG505 SOSIP.664 trimers may occur *in vivo* due to proteolysis by cells, tissue proteases, or other stresses. This may result in the decreased availability of intact BG505 SOSIP.664 trimers over an extended period after the bolus injection. In an attempt to sustain the availability of intact BG505 SOSIP.664 trimers during the GC response, we sequestered trimers in osmotic pumps that allowed for their slow delivery over many days. Negative-stain electron microscopy of BG505 SOSIP.664 trimers showed that 95% of trimers were in a closed conformation after being placed in osmotic pumps at 37°C (body temperature) for 14 days (see Fig. S7A in the supplemental material).

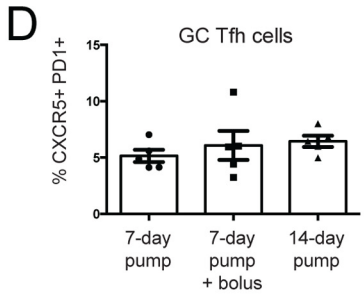
Three groups of mice were immunized with BG505 SOSIP.664 trimers: (i) group 1, delivery for 7 days via slow-release pumps; (ii) group 2, bolus injection and delivery for 7 days via slow-release pumps; (iii) group 3, delivery for 14 days via slow-release pumps. Pumps were inserted during each immunization at weeks 0, 8, and 19. At 4 weeks after the 3rd immunization, the frequencies of GC B cells and GC Tfh cells were similar among the three groups (Fig. 2B to E). However, the trimer-specific serum IgG titers differed. Thus, the lowest titers were found in the 7-day slow-release group (group 1), while in both of the other two groups the titers were ~10-fold higher (Fig. 2F). Hence, increasing the period of slow release or providing a supplemental bolus immunization increases the overall IgG response to the trimer. Of note, the anti-

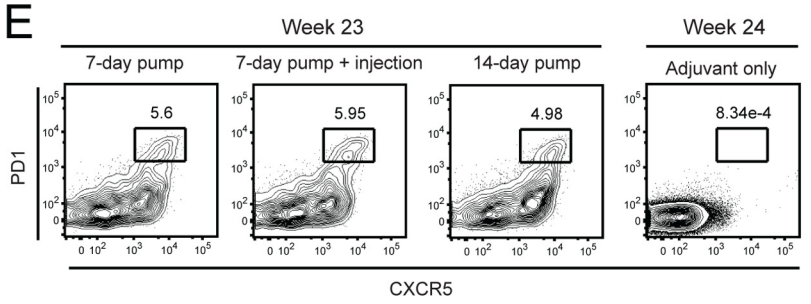
FIG 1 BG505 SOSIP.664 trimers are immunogenic and elicit Tier-1 neutralizing antibodies in mice. (A) BALB/c mice were immunized at weeks 0, 4, and 16 with 10, 10, and 20 μg of BG505 SOSIP trimers per mouse, respectively, in 0.2 μg of AbISCO-100 per mouse via footpads. Mouse serum was collected before and after immunizations as indicated. (B) GC B cell frequencies within the $CD19^+$ $CD4^-$ $CD8^-$ $CD11c^-$ F4/80$^-$ Gr-1^- B cell population in draining popliteal lymph nodes (PLNs) at week 18 (2 weeks after 3rd immunization) and week 0. (C) Fluorescence-activated cell sorter (FACS) plots of GC B cell frequencies gated on B cells at week 0 and week 18. Shown are frequencies of BG505 probe-specific GC B cells gated on Fas^+ $GL7^+$ $CD19^+$ $CD4^-$ $CD8^-$ $CD11c^-$ F4/80$^-$ Gr-1^- B cells. (D) GC Tfh cell frequencies within the $CD4^+$ $CD44^+$ $CD62L^-$ $CD19^-$ CD4 T cell population in draining PLNs at week 18 (2 weeks after 3rd immunization) and week 0. (E) FACS plots of GC Tfh cell frequencies gated on activated CD4 T cells at week 0 and week 18. (F) Kinetic of BG505 gp140-specific IgG in mouse serum. (G) Tier-1 HIV-1, Tier-2 HIV-1, and control VSV pseudovirus neutralization titers were measured at week 18 using mouse serum IgG from BG505-immunized mice compared to control mice receiving adjuvant alone or immunization with an irrelevant antigen, KLH. Human CD4 IgG2 was used as a positive control for binding the Tier-1 and Tier-2 HIV-1. (H) Data from neutralization assays showing percent inhibition of pseudovirus infection of TZM-bl cells over a series of dilution factors. Dilution factors of purified mouse IgG were calculated to represent dilution factors of original serum IgG. (I) Tier-1 and Tier-2 neutralization titers were assayed using purified mouse IgG from various immunization strategies. Strategies shown include the use of different mouse strains, antigen dosing, and adjuvants and the use of drug treatments. Data shown are from immunization strategies marked with an asterisk in Tables S1 to S6 in the supplemental material. (J) Validation of V3 Fc competition ELISA. V3 Fc incubation with V3-specific MAb 14e reduces BG505 SOSIP.664 trimer binding titers by ~5,000-fold. V3 Fc incubation with CD4 binding site-specific bnAb VRC01 shows negligible reduction in BG505 SOSIP.664 binding titers. (K) V3 Fc competition ELISA shows a 10-fold drop in BG505 SOSIP.664 trimer-specific IgG titers in mouse serum after incubation with V3 Fc.

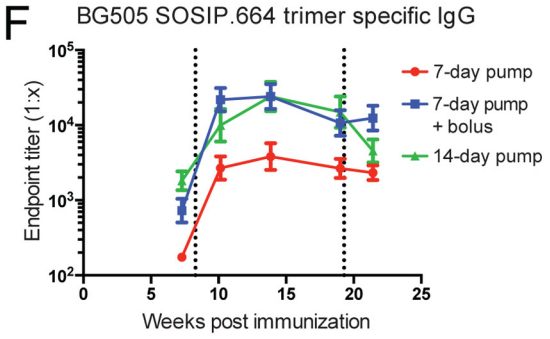


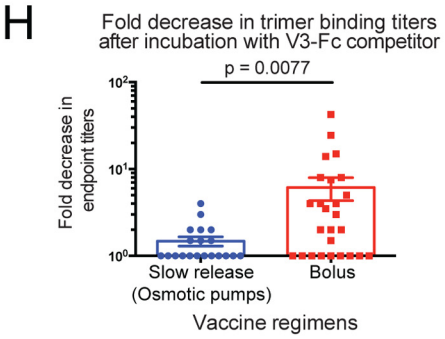


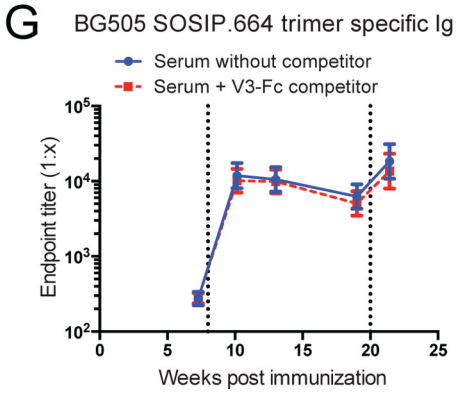












I

		IC50 (1:x)			
		Tier 2 Clade A	Tier 1 Clade B		Control
		BG505.T332N	MN	SF162	VSV
7-day pumps (m12+m13)	Week 23	<6	12	22	<6
Adjuvant only	Week 26	<8	<8	<8	<8
CD4IgG2	(ug/ml)	16	<0.05	0.16	>50

FIG 2 Antigen delivery via osmotic pumps reduces the immunodominant response to the V3 loop. (A) 129/Sv mice were immunized at weeks 0, 8, and 19 with BG505 SOSIP.664 trimers in Iscomatrix adjuvant in osmotic pumps. Group 1 received 7-day slow-release pumps containing 50 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix , group 2 received 7-day slow-release pumps containing 50 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix plus a bolus injection of 20 μg BG505 SOSIP.664 trimers in 0.5 U of Iscomatrix , and group 3 received 14-day slow-release pumps containing 100 μg BG505 SOSIP trimers in 0.5 U of

trimer IgG response was much less dominated by anti-V3 Abs when the trimers were delivered via the osmotic pump than with bolus immunizations using various vaccine strategies (Fig. 2H; also see Tables S1 to S6 in the supplemental material). Therefore, delivering trimers more slowly and in a sustained manner markedly reduced the Ab response to the V3 region. The reduction in the amount of anti-V3 Abs in the slow delivery groups was correlated with a decrease in the Tier-1 nAb titers, suggesting that V3-specific Abs mediated the Tier-1 virus neutralization (Fig. 2I; also see Fig. S8A). However, autologous Tier-2 nAbs still were not detected in any of the sera, irrespective of the trimer delivery method (Fig. 2I; also see Fig. S8A). Thus, while the V3 region of the trimers normally is immunodominant in mice, reducing its immunogenicity via a slow delivery method was not sufficient to allow the mice to generate autologous Tier-2 nAbs.

Dissociation kinetics and titers of trimer-specific mouse IgG. We next explored whether the lack of autologous Tier-2 nAbs in mice was attributable to suboptimal Ab dissociation kinetics or to inadequate titers of IgG Abs. Dissociation kinetics of Ab binding was evaluated by the use of an Octet biosensor. In control experiments, Octet biosensors with BG505 SOSIP.664 trimers were bound by multiple bnAbs against different epitopes (Fig. 3A). Importantly, the trimers were not bound by various non-nAbs, including the V3-specific Abs 19b, 14e, and 447-52-d (Fig. 3B). This solution-phase antigenicity data set confirmed that Octet biosensor-bound trimer immunogens were in the same native-like conformation reported previously using a binding kinetics assay with surface plasmon resonance (SPR) (27). Additionally, a titration of the bnAb 3BC315 showed that the off-rate constants (k_{off}) were independent of the Ab concentration (Fig. 3C and D), demonstrating that off-rate constants could be measured without information on the concentrations of distinct Abs in the polyclonal mouse IgG sample.

Having validated the assay, we compared the average off-rate constants for various immunized mouse IgG samples versus potent bnAbs (Fig. 3E to G). The trimer-reactive mouse IgG had off-rate constants comparable to those for highly potent human bnAbs, such as PGT151 (Fig. 3H). Once bound, mouse Abs dissociate slowly from the BG505 SOSIP.664 trimers, implying that the inability to neutralize the autologous BG505.T332N virus is unlikely to be rooted in suboptimal dissociation kinetics. These studies provide further support for the argument that the lack of autologous Tier-2 nAbs in the mice is not attributable to an inherently weak overall antibody response to the trimer immunogen.

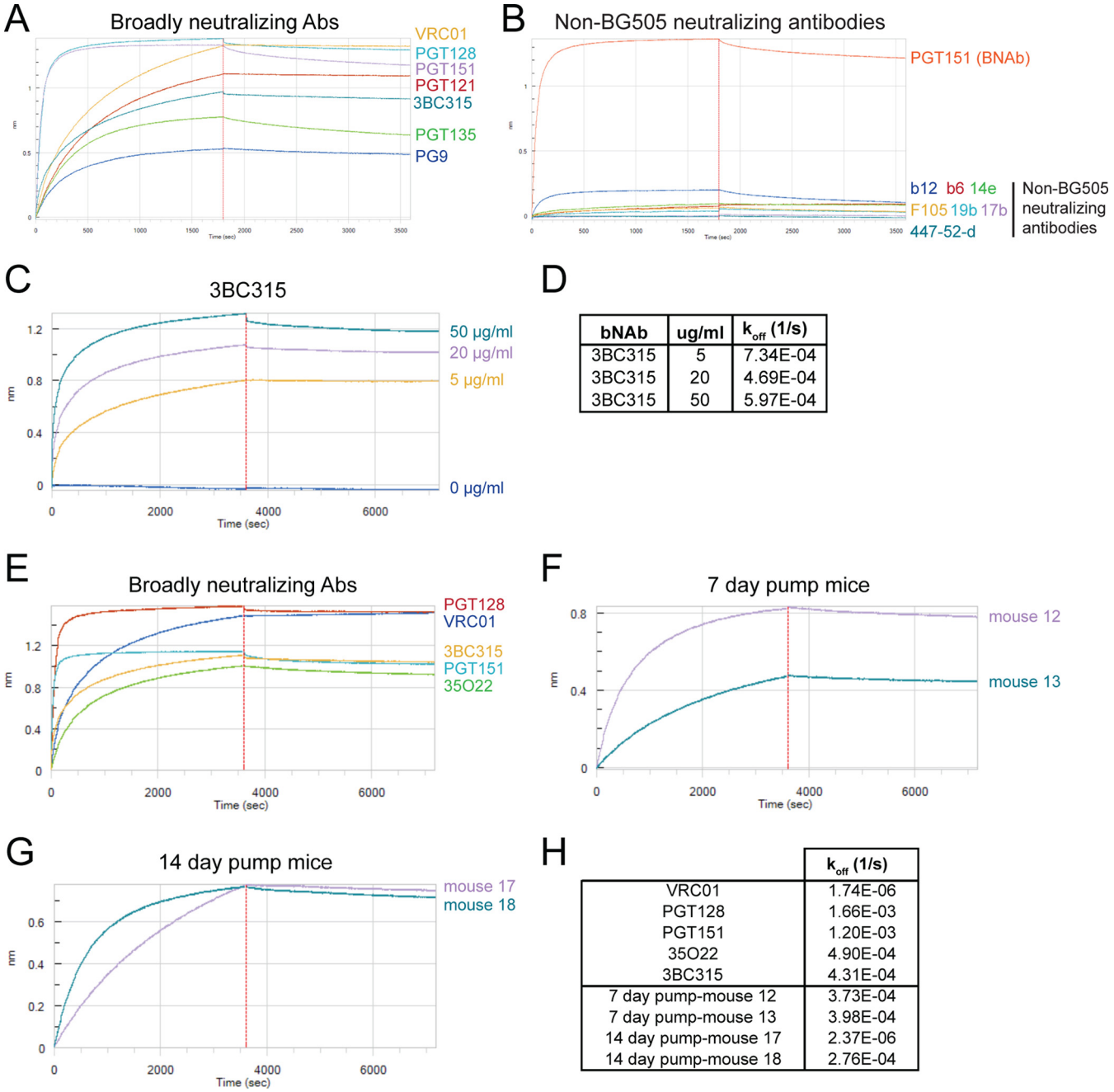
Mouse serum IgG binds to the base of soluble SOSIP trimers. Given the generation of high-titered, trimer-specific mouse Abs with slow dissociation, it was not known why these Abs did not neutralize the autologous Tier-2 BG505 virus. Our next hypothesis to account for the absence of autologous Tier-2 nAbs centered on the epitope specificities of the mouse IgG response. An Ab competition assay was used to explore what areas of the trimer were bound by the mouse anti-trimer IgG. The assay is based on quantifying the ability of an IgG sample to inhibit the trimer binding of various bnAbs specific to well-defined epitopes located on five different conserved regions of the trimer using Octet biosensors. The binding of bnAbs was impeded by other bnAbs to overlapping or proximal epitopes (Fig. 4A). For example, the binding of PGT128 to its high-mannose patch epitope was blocked by PGT126, which binds to an overlapping high-mannose patch epitope, but not by PGT145, which recognizes a more distant site involving V1V2-glycans (Fig. 4A).

The bnAbs used to evaluate the epitope specificities of the mouse sera were PGT145, specific for the apex (V1V2 Asn160) (63), PGT128, specific for the high-mannose patch V3 glycan (29), PGT135, specific for a lower region of the high-mannose patch (64), VRC01, specific for the CD4 binding site (65), PGT151 (66) and 35O22 (5), specific for the gp120-gp41 interface, and 3BC315, specific for gp41 (30) (J. H. Lee, submitted for publication). Mouse IgG Abs from the osmotic pump experiments were examined using the competition assay. The mouse IgG samples competed most strongly with bnAb 3BC315 or 35O22, which bound to epitopes located near the bottom of the trimer (Fig. 4B and C). When the competition assay was carried out in the reverse format (i.e., assessing whether bnAbs could inhibit mouse serum IgG binding to the trimer) at saturating levels of bnAbs, 35O22 and 3BC315 inhibited mouse IgG binding to the trimer by 37 to 74% (Fig. 5A and B). However, unlike 35O22 and 3BC315, the mouse anti-trimer IgG fails to neutralize BG505.T332N. Therefore, it is reasonable to conclude that mouse anti-trimer IgGs bind close to the bottom of soluble trimers, possibly at neo-epitopes in the C terminus or at conformational disturbances near the C terminus. Ab binding epitopes that are not present or accessible on the BG505 virion would not neutralize virus. Modeling BG505 SOSIP.664 with full glycans shows that the glycan shield protects most surface envelope trimers (Fig. 6). However, upon closer examination, it becomes apparent that the bottom of the soluble trimer is exposed and not shielded by glycans (Fig. 6, right). The base is the largest contiguous protein surface of the BG505 SOSIP.664 trimer and may be much more readily bound by murine B cells.

DISCUSSION

Most HIV-1-infected people develop Tier-2 nAbs of narrow specificity, and a subset of these individuals later develop a broader nAb response (67–70). However, inducing Tier-2 nAbs even of narrow specificity (e.g., autologous) in a protein vaccination by

Iscomatrix. Mouse serum was collected before and after immunizations as indicated. (B) GC B cell frequencies within the $CD19^+$ $CD4^-$ $CD8^-$ $CD11c^-$ $F4/80^-$ $Gr\text{-}1^-$ B cell population in draining brachial lymph nodes (BLNs) at week 23 (4 weeks after 3rd immunization). (C) FACS plots of GC B cell frequencies gated on B cells at week 23 in BG505 and pump-immunized mice. GC B cell frequencies at week 24 in mice immunized with adjuvant alone are shown. (D) GC Tfh cell frequencies within the $CD4^+$ $CD44^+$ $CD62L^-$ $CD19^-$ CD4 T cell population in draining BLNs at week 23 (4 weeks after 3rd immunization). (E) FACS plots of GC Tfh cell frequencies gated on activated CD4 T cells at week 23 in BG505 SOSIP.664 trimer and pump-immunized mice. GC Tfh cell frequencies at week 24 in mice immunized with adjuvant alone. (F) Kinetic of BG505 SOSIP.664 trimer-specific IgG in mouse serum. (G) Kinetics of BG505 SOSIP.664 trimer binding IgG titers from mice that received 7-day pumps plus bolus immunization. Preincubation of serum with V3-Fc showed a 1.34-fold drop in BG505 gp140-specific IgG titers in mouse serum after incubation with V3 Fc. (H) Fold decrease in BG505 SOSIP.664 trimer binding IgG titers after incubation with V3-Fc. (I) Tier-1 HIV-1, Tier-2 HIV-1, and control VSV pseudovirus neutralization titers were measured at week 23 using mouse serum IgG from mice immunized with 7-day slow-release pumps plus bolus injection. Mouse serum IgG from a mouse receiving adjuvant only was used as a negative control. Human CD4 IgG2 was used as a positive control for binding Tier-1 and Tier-2 HIV-1.



bNAb	ug/ml	k_{off} (1/s)
3BC315	5	7.34E-04
3BC315	20	4.69E-04
3BC315	50	5.97E-04

H

	k_{off} (1/s)
VRC01	1.74E-06
PGT128	1.66E-03
PGT151	1.20E-03
35O22	4.90E-04
3BC315	4.31E-04
7 day pump-mouse 12	3.73E-04
7 day pump-mouse 13	3.98E-04
14 day pump-mouse 17	2.37E-06
14 day pump-mouse 18	2.76E-04

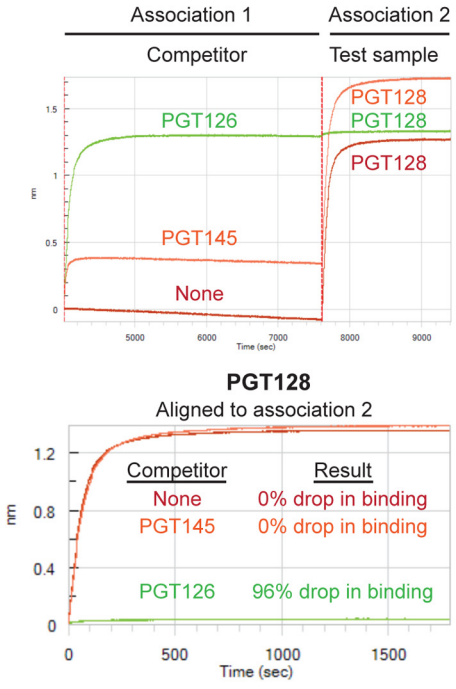
FIG 3 Dissociation rate kinetics as measured by the biosensor binding kinetics assay (Octet). (A) Association and dissociation of bnAbs to BG505 SOSIP.664 trimers. (B) Association and dissociation of non-BG505 neutralizing antibodies to BG505 SOSIP.664 trimers. PGT151 is a BG505 neutralizing Ab used as a comparison. (C) Association and dissociation of 3BC315 at different concentrations. (D) Average off-rate constants of 3BC315 at different concentrations. (E) Association and dissociation of 3BC315 and 35O22 compared to those of other bnAbs. (F) Association and dissociation of purified IgG from mice receiving 7-day pumps. (G) Association and dissociation of purified IgG from mice receiving 14-day pumps. (H) Average off-rate constants of purified IgG from mice receiving immunization with pumps compared to average off rates of bnAbs.

Env protein immunization has proven difficult. Therefore, the evaluation of the latest Env-based immunogens in animal models is an essential part of the vaccine development process, particularly to gauge the ability to induce Tier-2 nAbs. The BG505 SOSIP.664 trimers have very recently been shown to induce a strong and consistent autologous Tier-2 nAb response in rabbits and a weaker response in macaques. A second native-like trimer, B41 SOSIP.664, also is capable of inducing autologous Tier-2 nAbs in rabbits (33). Therefore, we evaluated the same BG505 SOSIP.664 trimers in mice because of the immunological tools available in the mouse model.

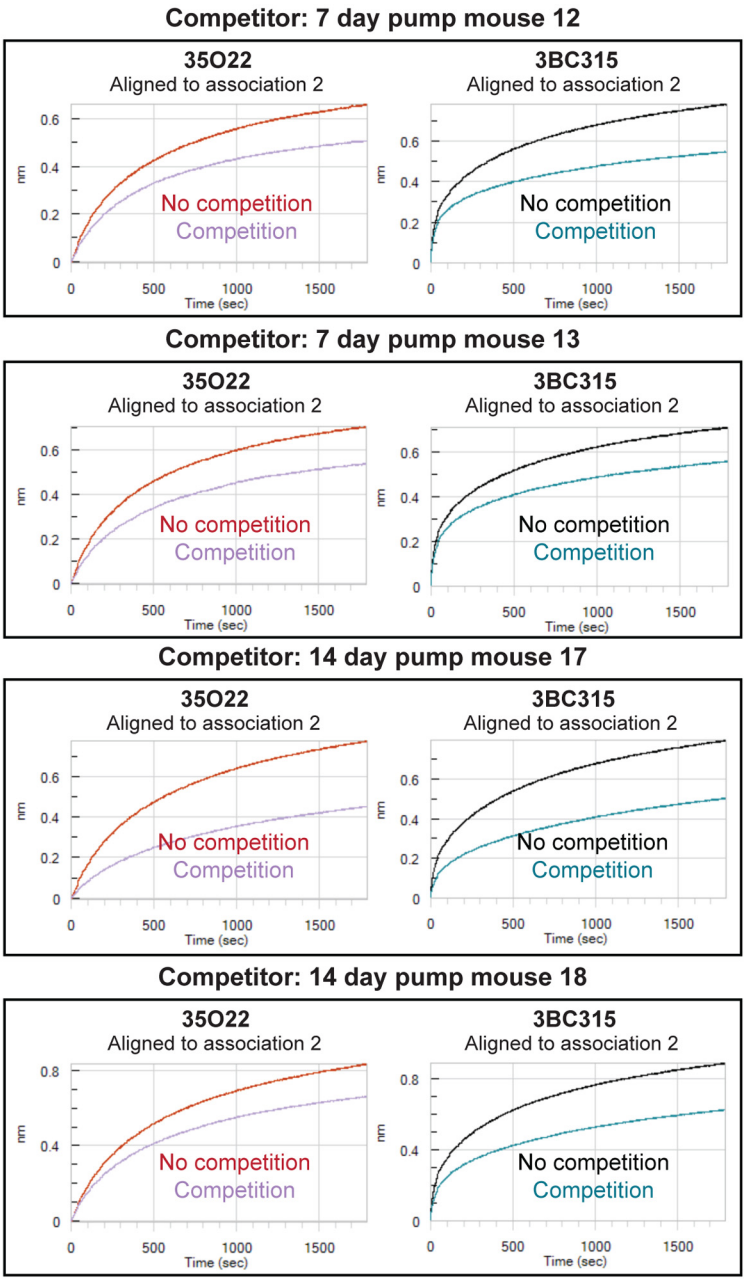
In an extensive series of experiments that involved a multimonth immunization regimen with BG505 SOSIP.664 trimers, conventional mice generated Tier-1 nAbs but not autologous

A

BNAb	Epitope specificity
PGT126	V3-glycan
PGT128	V3-glycan
PGT145	V1V2-glycan



B



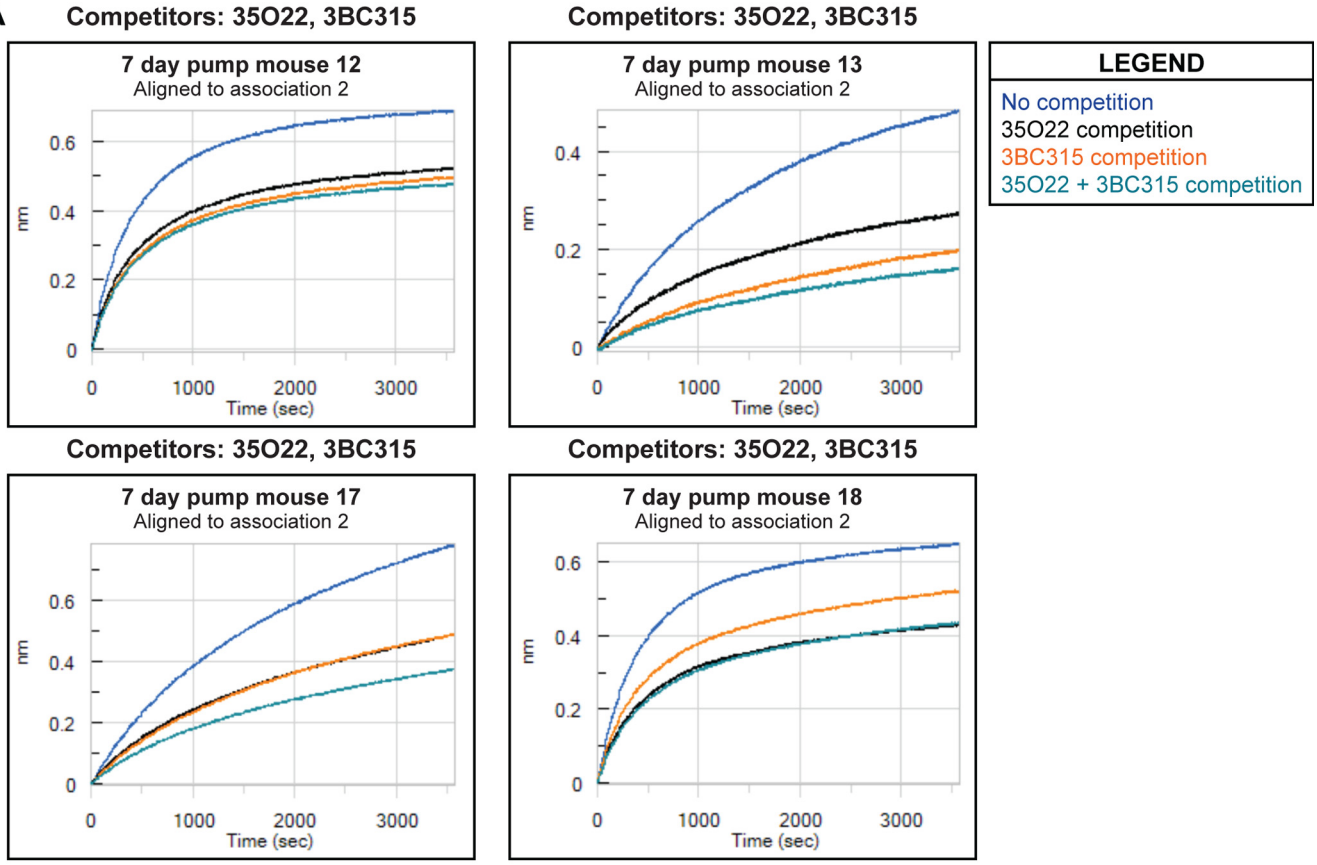
C

	V1V2 glycan	Oligomannose patch		CD4bs	gp120-gp41		gp41
		V3 glycan	OD-glycan				
	PGT145	PGT128	PGT135	VRC01	PGT151	35O22	3BC315
7 day pump (m12)	9	14	10	1	17	22	29
7 day pump (m13)	10	-2	17	0	7	26	24
14 day pump (m17)	1	-1	23	13	3	51	45
14 day pump (m18)	17	10	15	6	7	22	31

75-100% drop in binding
50-74% drop in binding
20-49% drop in binding
0-19% drop in binding

FIG 4 Mouse serum IgG competes for binding to BG505 SOSIP.664 trimers with bnAbs specific to the base of Env trimers. (A) Validation of antibody competition assay. bnAbs and epitope specificities are indicated. Association of PGT128 (Association 2) is shown after no competition (red), competition with PGT125 (orange), or competition with PGT126 (green). Alignment of association of PGT128 with or without competition shows the percent drop in binding. (B) Antibody competition assay was performed using serum IgG from pump-immunized mice as competitors. A drop in association of 35O22 and 3BC315 in the presence of competing mouse serum IgG is shown. (C) Percent drop in binding of bnAbs to BG505 SOSIP.664 trimers in the presence of serum IgG from pump-immunized mice.

A



B

Competitor	7 day pump		14 day pump	
	m12	m13	m17	m18
35O22	29	51	41	40
3BC315	30	62	40	22
35O22+3BC315	37	74	58	39

75-100% drop in binding
50-74% drop in binding
25-49% drop in binding
25-49% drop in binding

FIG 5 BnAbs 35O22 and 3BC315 compete with mouse IgG for binding to BG505 SOSIP.664 trimers. (A) Antibody competition assay was performed using 35O22 and 3BC315 as competitors. A drop in association of serum IgG from pump-immunized mice in the presence of 35O22, 3BC315, or 35O22 and 3BC315 is shown. (B) Percent drop in binding of serum IgG from pump-immunized mice to BG505 SOSIP.664 trimers in the presence of 35O22 and 3BC315.

Tier-2 nAbs. Mice generated Abs specific to the immunodominant V3 region of the gp120 subunit, similar to rabbits. Abs to these V3 epitopes can strongly neutralize Tier-1 viruses, but they are rarely active against Tier-2 viruses, including the autologous BG505.T332N strain (19, 33). Hence, the V3 region of first-generation BG505 SOSIP.664 trimers is strongly immunogenic in animals and may be caused by V3 loop exposure via natural conformational changes of the trimer or by proteolytic events in tissues. In support of the latter hypothesis, sustained delivery of trimers via osmotic pumps significantly reduced the V3-specific component of the overall Ab response. However, this reduction in V3 loop-specific Abs was not accompanied by the appearance of autologous Tier-2 nAbs. To understand why trimer-specific Abs did not neutralize the autologous BG505 virus, Ab competition assays were performed to assess areas of the trimer that were bound by mouse Abs. These competition assays indicate that mouse Abs bound near the base of soluble BG505 SOSIP.664 trimers, potentially at neo-epitopes that were created as a consequence of making these trimers soluble. As the corresponding region either does not exist on the native virion-associated trimer or is occluded by the virion membrane, Abs to this site are not capable of neutralizing the virus.

When initiating this study, we were aware that mice often are considered to have much shorter heavy-chain CDRH3 lengths than humans, macaques, or rabbits (71–74); thus, they may be at a disadvantage for raising Abs capable of penetrating the trimer's glycan shield. However, there are many examples of both strain-specific Tier-2 HIV-1 nAbs and bnAbs that have shorter CDRH3s (75), which implies that a long CDRH3 is not a mandatory requirement for an Ab to neutralize HIV-1. Thus, although we did not expect that mice would develop bnAbs like the PG9-class Abs that do have very long CDRH3 elements, we considered it possible that autologous Tier-2 nAbs would be raised against other epitopes for which long CDRH3s are not required. However, it appears that it is very difficult for mice to make Tier-2 nAbs. Autologous Tier-2 nAbs elicited in BG505 SOSIP.664 trimer-im-

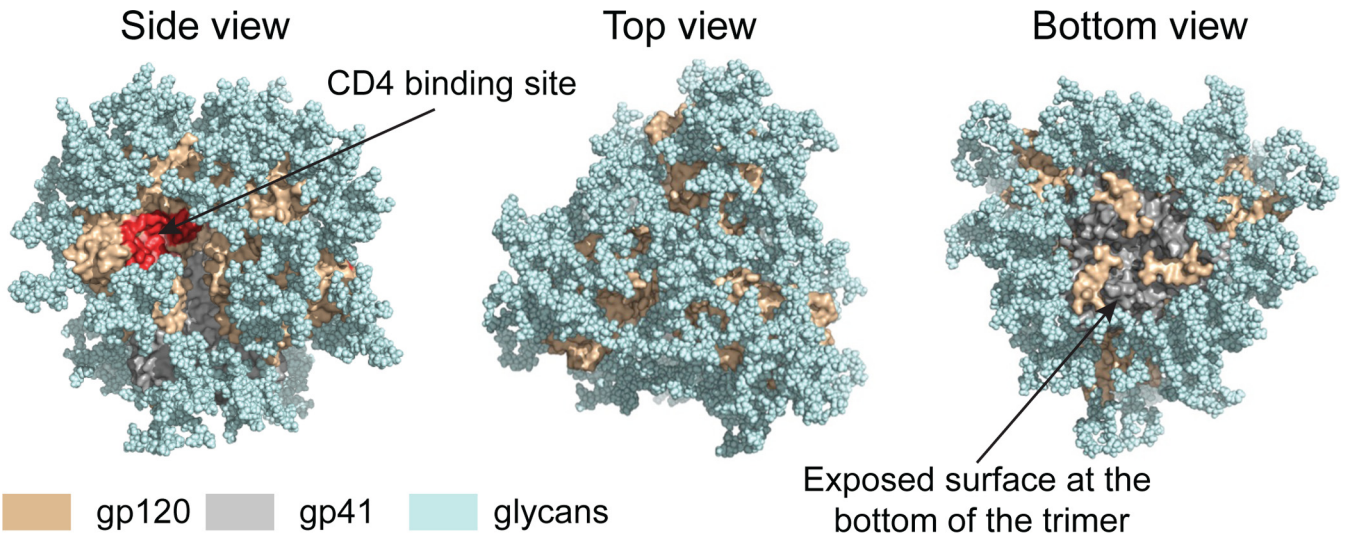


FIG 6 Structural model of soluble BG505 SOSIP.664 trimers. Shown are the side view, top view, and bottom view of a soluble BG505 SOSIP.664 trimer (4TVP.pdb [Pancera14]) with glycans (aqua) surrounding the gp120 (tan) and gp41 (gray) domains of the trimer protein. Glycans were modeled using Glyprot (http://www.glycosciences.de/modeling/glyprot/) according to Table 1 and Table S2 of Guttman et al. (91).

munized rabbits recognize glycan-dependent epitopes, and these Tier-2 nAbs may at least partially penetrate gaps in the glycan shield (33). A glycan shield-penetrative ability also has been reported recently for Tier-2 nAbs elicited in rabbits after immunization with virus-like particles (VLPs) expressing native-like, SOS-stabilized trimers (76). Therefore, mice may somehow be at a severe disadvantage for inducing Abs that can penetrate the glycan shield.

There is precedence for reduced responses to highly glycosylated proteins in mice. The lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) is highly glycosylated (77), and mice either fail to develop nAbs to GP or they do so very slowly compared to less glycosylated proteins, like G proteins from VSV (78–82). Therefore, it seems possible that there is a strong selection pressure in mice against B cells that bind glycoprotein epitopes (83). Therefore, to examine the response to native-like HIV-1 Env trimer immunogens, it is important to employ animal models with relevant B cell receptor (BCR) repertoires that can bind to the highly glycosylated epitopes of the HIV-1 Env trimer. These include humanized mouse models with individual transgenic human germ line BCRs (84, 85), transgenic BCRs of human bnAbs (85), and humanized mice with polyclonal human BCR repertoires (86–88).

In this study, we used BG505 SOSIP.664 trimer immunogens because they are more native-like in conformation (27–29) than previous versions of Env trimers. We rationalized that the presentation of more native-like epitopes on BG505 SOSIP.664 trimers would be useful for generating Tier-2 nAbs. However, because BG505 SOSIP.664 trimers are more native-like, they may be less immunogenic due to glycan shielding of protein epitopes in a manner similar to that of trimers on viruses. Here, we show that BG505 SOSIP.664 trimer immunogens are immunogenic *in vivo* and generate frequencies of Tfh cells and GC B cells after immunization that are similar to those of YU2 gp140-F. However, our study suggests that mice have a restricted BCR repertoire that poorly recognizes glycan-containing epitopes. Therefore, although we find Tfh cell and germinal center responses in BG505 SOSIP.664 trimer-immunized mice, if naive B cells of appropriate Tier-2-neutralizing epitope specificity do not exist in the repertoire, then Tfh cells will only be able to help the B cells of the nonneutralizing and Tier-1-neutralizing specificities. In contrast, in the context of other animal models (i.e., rabbits and macaques) that can elicit Tier-2 nAbs, Tfh cells may have a substantial impact on the generation of high-affinity Tier-2 nAbs given their role in GC B cell selection (89) and extent of somatic hypermutation per selection cycle (16, 90). Thus, it will be important to continue understanding the role of Tfh cells in the generation of high-affinity Tier-2 nAbs and bnAbs.

These results can be used to inform immunogen design and delivery. These data suggest that the base of the BG505 SOSIP.664 trimer is an immunodominant epitope region for mouse B cell responses, perhaps because this region is the largest area of the trimer that is not shielded by glycans (Fig. 6). The generation of antibodies near the trimer base most likely is not specific to mice, given the large exposed proteinaceous surface of the trimer base. Cross-competition ELISAs have been published for serum Ab responses by BG505 Env trimer-immunized rabbits (33). Serum Ab from most BG505 Env trimer-immunized rabbits competed with bnAbs 35O22 and 3BC315 for trimer binding. However, given that rabbits make autologous Tier-2 nAbs, crossblocking epitope mapping experiments cannot directly discern if rabbit Abs bind to nonneutralizing areas at the trimer base, similar to the mice, or if all of the detected rabbit Ab trimer binding reflects neutralizing antibodies. Specific epitopes at the base of soluble trimers may not be relevant for neutralization due to their occlusion by the viral membrane, and it is possible that these epitopes could be distractive to responses against more favorable epitopes. If future work indicates that such immune distraction mechanisms exist, then it could be useful to occlude the base of the trimer to reduce its immunogenicity. For example, the presentation of trimers on nanoparticle configurations could occlude the base of the trimer and enhance B cell recruitment through increased valency. Moreover, sustained release of the trimers through sustained release platforms may enhance GC responses and focus B cell responses

on closed trimer epitopes. Furthermore, additional mutations can be made to BG505 SOSIP.664 trimers to further stabilize the closed trimer and reduce the exposure of Tier-1 neutralizing epitopes like the V3 loop. While mutations introduced into BG505 SOSIP.664 trimers increase trimer stabilization while retaining bnAb epitopes, antigenicity is not the same as immunogenicity, and it is important to test new Env trimer immunogens *in vivo* for the ability to elicit autologous and heterologous Tier-2 neutralizing Abs. These immunogen design strategies and immunological approaches can be harnessed to support the evolution of nAb potency and breadth. Both disciplines have key roles to play in the eventual development of an HIV-1 vaccine.

ACKNOWLEDGMENTS

This work was supported by Scripps CHAVI-ID (UM1-Al100663). This work was partially funded by IAVI with the generous support of U.S. AID and the Bill & Melinda Gates Foundation; a full list of IAVI donors is available at www.iavi.org.

We thank Richard Wyatt and Richard Wilson for generously providing YU2 gp140-F trimers. We thank CSL for Iscomatrix adjuvant and Aerpio Therapeutics for AKB-4924.

REFERENCES

1. **Walker LM, Phogat SK, Chan-Hui P-Y, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Protocol Principal Investigators Group, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR.** 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science **326:**285–289. http://dx.doi.org/10.1126/science.1178746.
2. **Wu X, Yang Z-Y, Li Y, Hogerkorp C-M, Schief WR, Seaman MS, Zhou T, Schmidt SD, Wu L, Xu L, Longo NS, McKee K, O'Dell S, Louder MK, Wycuff DL, Feng Y, Nason M, Doria-Rose N, Connors M, Kwong PD, Roederer M, Wyatt RT, Nabel GJ, Mascola JR.** 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science **329:**856–861. http://dx.doi.org/10.1126/science.1187659.
3. **Falkowska E, Ramos A, Feng Y, Zhou T, Moquin S, Walker LM, Wu X, Seaman MS, Wrin T, Kwong PD, Wyatt RT, Mascola JR, Poignard P, Burton DR.** 2012. PGV04, an HIV-1 gp120 CD4 binding site antibody, is broad and potent in neutralization but does not induce conformational changes characteristic of CD4. J Virol **86:**4394–4403. http://dx.doi.org/10.1128/JVI.06973-11.
4. **Wu X, Zhou T, Zhu J, Zhang B, Georgiev I, Wang C, Chen X, Longo NS, Louder M, McKee K, O'Dell S, Perfetto S, Schmidt SD, Shi W, Wu L, Yang Y, Yang Z-Y, Yang Z, Zhang Z, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Simek M, Burton DR, Koff WC, Doria-Rose NA, Connors M, Comparative Sequencing Program NISC, Mullikin JC, Nabel GJ, Roederer M, Shapiro L, Kwong PD, Mascola JR.** 2011. Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing. Science **333:**1593–1602. http://dx.doi.org/10.1126/science.1207532.
5. **Huang J, Kang BH, Pancera M, Lee JH, Tong T, Feng Y, Georgiev IS, Chuang G-Y, Druz A, Doria-Rose NA, Laub L, Sliepen K, van Gils MJ, la Peña de AT, Derking R, Klasse PJ, Migueles SA, Bailer RT, Alam M, Pugach P, Haynes BF, Wyatt RT, Sanders RW, Binley JM, Ward AB, Mascola JR, Kwong PD, Connors M.** 2014. Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface. Nature **515:**138–142. http://dx.doi.org/10.1038/nature13601.
6. **Moldt B, Rakasz EG, Schultz N, Chan-Hui P-Y, Swiderek K, Weisgrau KL, Piaskowski SM, Bergman Z, Watkins DI, Poignard P, Burton DR.** 2012. Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. Proc Natl Acad Sci U S A **109:**18921–18925. http://dx.doi.org/10.1073/pnas.1214785109.
7. **Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK, Parren PWHI, Marx PA, Burton DR.** 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med **15:**951–954. http://dx.doi.org/10.1038/nm.1974.
8. **Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, Koff WC, Watkins DI, Burton DR.** 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog **5:**e1000433. http://dx.doi.org/10.1371/journal.ppat.1000433.
9. **Shingai M, Donau OK, Plishka RJ, Buckler-White A, Mascola JR, Nabel GJ, Nason MC, Montefiori D, Moldt B, Poignard P, Diskin R, Bjorkman PJ, Eckhaus MA, Klein F, Mouquet H, Cetrulo Lorenzi JC, Gazumyan A, Burton DR, Nussenzweig MC, Martin MA, Nishimura Y.** 2014. Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. J Exp Med **211:**2061–2074. http://dx.doi.org/10.1084/jem.20132494.
10. **Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, Wang S-K, Ramos A, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong C-H, Phogat S, Wrin T, Simek MD, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P.** 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature **477:**466–470. http://dx.doi.org/10.1038/nature10373.
11. **Klein F, Diskin R, Scheid JF, Gaebler C, Mouquet H, Georgiev IS, Pancera M, Zhou T, Incesu R-B, Fu BZ, Gnanapragasam PNP, Oliveira TY, Seaman MS, Kwong PD, Bjorkman PJ, Nussenzweig MC.** 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. Cell **153:**126–138. http://dx.doi.org/10.1016/j.cell.2013.03.018.
12. **West AP, Jr, Scharf L, Scheid JF, Klein F, Bjorkman PJ, Nussenzweig MC.** 2014. Structural insights on the role of antibodies in HIV-1 vaccine and therapy. Cell **156:**633–648. http://dx.doi.org/10.1016/j.cell.2014.01.052.
13. **Sok D, Laserson U, Laserson J, Liu Y, Vigneault F, Julien J-P, Briney B, Ramos A, Saye KF, Le K, Mahan A, Wang S, Kardar M, Yaari G, Walker LM, Simen BB, John EPS, Chan-Hui P-Y, Swiderek K, Kleinstein SH, Alter G, Seaman MS, Chakraborty AK, Koller D, Wilson IA, Church GM, Burton DR, Poignard P.** 2013. The Effects of Somatic Hypermutation on Neutralization and Binding in the PGT121 Family of Broadly Neutralizing HIV Antibodies. PLoS Pathog **9:**e1003754. http://dx.doi.org/10.1371/journal.ppat.1003754.
14. **Victora GD, Nussenzweig MC.** 2012. Germinal centers. Annu Rev Immunol **30:**429–457. http://dx.doi.org/10.1146/annurev-immunol-020711-075032.
15. **Crotty S.** 2014. T follicular helper cell differentiation, function, and roles in disease. Immunity **41:**529–542. http://dx.doi.org/10.1016/j.immuni.2014.10.004.
16. **Gitlin AD, Shulman Z, Nussenzweig MC.** 2014. Clonal selection in the germinal centre by regulated proliferation and hypermutation. Nature **509:**637–640. http://dx.doi.org/10.1038/nature13300.
17. **Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, Ambrozak DR, Sandler NG, Timmer KJ, Sun X, Pan L, Poholek A, Rao SS, Brenchley JM, Alam SM, Tomaras GD, Roederer M, Douek DC, Seder RA, Germain RN, Haddad EK, Koup RA.** 2012. CD4 T follicular helper cell dynamics during SIV infection. J Clin Investig **122:**3281–3294. http://dx.doi.org/10.1172/JCI63039.
18. **Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, Su LF, Cubas R, Davis MM, Sette A, Haddad EK, International AIDS Vaccine Initiative Protocol Principal Investigators Committee, Poignard P, Crotty S.** 2013. Human circulating PD-1+ CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity **39:**758–769. http://dx.doi.org/10.1016/j.immuni.2013.08.031.
19. **Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert PB, Self SG, Korber BT, Montefiori DC, Mascola JR.** 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J Virol **84:**1439–1452. http://dx.doi.org/10.1128/JVI.02108-09.
20. **Moore JP, Ho DD.** 1995. HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. AIDS **9**(Suppl A):S117–S136.
21. **DeCamp A, Hraber P, Bailer RT, Seaman MS, Ochsenbauer C, Kappes J, Gottardo R, Edlefsen P, Self S, Tang H, Greene K, Gao H, Daniell X, Sarzotti-Kelsoe M, Gorny MK, Zolla-Pazner S, LaBranche CC, Mascola JR, Korber BT, Montefiori DC.** 2014. Global panel of HIV-1 Env reference strains for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol **88:**2489–2507. http://dx.doi.org/10.1128/JVI.02853-13.
22. **Mascola JR, Montefiori DC.** 2010. The role of antibodies in HIV

vaccines. Annu Rev Immunol **28:**413–444. http://dx.doi.org/10.1146/annurev-immunol-030409-101256.
23. **Guttman M, Cupo A, Julien J-P, Sanders RW, Wilson IA, Moore JP, Lee KK.** 2015. Antibody potency relates to the ability to recognize the closed, pre-fusion form of HIV Env. Nat Commun **6:**6144. http://dx.doi.org/10.1038/ncomms7144.
24. **Bartesaghi A, Merk A, Borgnia MJ, Milne JLS, Subramaniam S.** 2013. Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy. Nat Struct Mol Biol **20:**1352–1357. http://dx.doi.org/10.1038/nsmb.2711.
25. **Gilbert P, Wang M, Wrin T, Petropoulos C, Gurwith M, Sinangil F, D'Souza P, Rodriguez-Chavez IR, DeCamp A, Giganti M, Berman PW, Self SG, Montefiori DC.** 2010. Magnitude and breadth of a nonprotective neutralizing antibody response in an efficacy trial of a candidate HIV-1 gp120 vaccine. J Infect Dis **202:**595–605. http://dx.doi.org/10.1086/654816.
26. **Montefiori DC, Karnasuta C, Huang Y, Ahmed H, Gilbert P, de Souza MS, McLinden R, Tovanabutra S, Laurence-Chenine A, Sanders-Buell E, Moody MA, Bonsignori M, Ochsenbauer C, Kappes J, Tang H, Greene K, Gao H, LaBranche CC, Andrews C, Polonis VR, Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Self SG, Berman PW, Francis D, Sinangil F, Lee C, Tartaglia J, Robb ML, Haynes BF, Michael NL, Kim JH.** 2012. Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials. J Infect Dis **206:**431–441. http://dx.doi.org/10.1093/infdis/jis367.
27. **Sanders RW, Derking R, Cupo A, Julien J-P, Yasmeen A, de Val N, Kim HJ, Blattner C, la Peña de AT, Korzun J, Golabek M, de los Reyes K, Ketas TJ, van Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP.** 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, Expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog **9:**e1003618. http://dx.doi.org/10.1371/journal.ppat.1003618.
28. **Lyumkis D, Julien J-P, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB.** 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science **342:**1484–1490. http://dx.doi.org/10.1126/science.1245627.
29. **Julien J-P, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA.** 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science **342:**1477–1483. http://dx.doi.org/10.1126/science.1245625.
30. **Derking R, Ozorowski G, Sliepen K, Yasmeen A, Cupo A, Torres JL, Julien J-P, Lee JH, van Montfort T, de Taeye SW, Connors M, Burton DR, Wilson IA, Klasse PJ, Ward AB, Moore JP, Sanders RW.** 2015. Comprehensive antigenic map of a cleaved soluble HIV-1 envelope trimer. PLoS Pathog **11:**e1004767. http://dx.doi.org/10.1371/journal.ppat.1004767.
31. **Sok D, van Gils MJ, Pauthner M, Julien J-P, Saye-Francisco KL, Hsueh J, Briney B, Lee JH, Le KM, Lee PS, Hua Y, Seaman MS, Moore JP, Ward AB, Wilson IA, Sanders RW, Burton DR.** 2014. Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex. Proc Natl Acad Sci U S A **111:**17624–17629. http://dx.doi.org/10.1073/pnas.1415789111.
32. **Pancera M, Zhou T, Druz A, Georgiev IS, Soto C, Gorman J, Huang J, Acharya P, Chuang G-Y, Ofek G, Stewart-Jones GBE, Stuckey J, Bailer RT, Joyce MG, Louder MK, Tumba N, Yang Y, Zhang B, Cohen MS, Haynes BF, Mascola JR, Morris L, Munro JB, Blanchard SC, Mothes W, Connors M, Kwong PD.** 2014. Structure and immune recognition of trimeric pre-fusion HIV-1 Env. Nature **514:**455–461. http://dx.doi.org/10.1038/nature13808.
33. **Sanders RW, van Gils MJ, Derking R, Sok D, Ketas TJ, Burger JA, Ozorowski G, Cupo A, Simonich C, Goo L, Arendt H, Kim HJ, Lee JH, Pugach P, Williams M, Debnath G, Moldt B, van Breemen MJ, Isik G, Medina-Ramírez M, Back JW, Koff WC, Julien J-P, Rakasz EG, Seaman MS, Guttman M, Lee KK, Klasse PJ, Labranche C, Schief WR, Wilson IA, Overbaugh J, Burton DR, Ward AB, Montefiori DC, Dean H, Moore JP.** 2015. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science **349:**aac4223. http://dx.doi.org/10.1126/science.aac4223.
34. **Liao H-X, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD, Fire AZ, Roskin KM, Schramm CA, Zhang Z, Zhu J, Shapiro L, Comparative Sequencing Program NISC, Mullikin JC, Gnanakaran S, Hraber P, Wiehe K, Kelsoe G, Yang G, Xia S-M, Montefiori DC, Parks R, Lloyd KE, Scearce RM, Soderberg KA, Cohen M, Kamanga G, Louder MK, Tran LM, Chen Y, Cai F, Chen S, Moquin S, Du X, Joyce MG, Srivatsan S, Zhang B, Zheng A, Shaw GM, Hahn BH, Kepler TB, Korber BTM, Kwong PD, Mascola JR, Haynes BF.** 2013. Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. Nature **496:**469–476. http://dx.doi.org/10.1038/nature12053.
35. **Streeck H, D'Souza MP, Littman DR, Crotty S.** 2013. Harnessing $CD4^+$ T cell responses in HIV vaccine development. Nat Med **19:**143–149. http://dx.doi.org/10.1038/nm.3054.
36. **Montefiori DC.** 2001. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. John Wiley & Sons, Inc., Hoboken, NJ.
37. **National Research Council.** 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
38. **Code of Federal Regulations.** 1989. Final rules: animal welfare act. 9 CFR parts 1, 2, and 3. U.S. Department of Agriculture, Washington, DC.
39. **Waggoner SN, Cornberg M, Selin LK, Welsh RM.** 2012. Natural killer cells act as rheostats modulating antiviral T cells. Nature **481:**394–398.
40. **Yang H, Yogeeswaran G, Bukowski JF, Welsh RM.** 1985. Expression of asialo GM1 and other antigens and glycolipids on natural killer cells and spleen leukocytes in virus-infected mice. Nat Immun Cell Growth Regul **4:**21–39.
41. **Chung NP, Matthews K, Kim HJ, Ketas TJ, Golabek M, de los Reyes K, Korzun J, Yasmeen A, Sanders RW, Klasse PJ, Wilson IA, Ward AB, Marozsan AJ, Moore JP, Cupo A.** 2014. Stable 293 T and CHO cell lines expressing cleaved, stable HIV-1 envelope glycoprotein trimers for structural and vaccine studies. Retrovirology **11:**33. http://dx.doi.org/10.1186/1742-4690-11-33.
42. **Forsell MNE, Dey B, Mörner A, Svehla K, O'Dell S, Hogerkorp C-M, Voss G, Thorstensson R, Shaw GM, Mascola JR, Karlsson Hedestam GB, Wyatt RT.** 2008. B cell recognition of the conserved HIV-1 co-receptor binding site is altered by endogenous primate CD4. PLoS Pathog **4:**e1000171. http://dx.doi.org/10.1371/journal.ppat.1000171.
43. **Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S.** 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity **34:**932–946. http://dx.doi.org/10.1016/j.immuni.2011.03.023.
44. **Yang Q, Li C, Wei Y, Huang W, Wang L-X.** 2010. Expression, glycoform characterization, and antibody-binding of HIV-1 V3 glycopeptide domain fused with human IgG1-Fc. Bioconjug Chem **21:**875–883. http://dx.doi.org/10.1021/bc9004238.
45. **Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312. http://dx.doi.org/10.1038/nature01470.
46. **Derdeyn CA, Decker JM, Sfakianos JN, Wu X, O'Brien WA, Ratner L, Kappes JC, Shaw GM, Hunter E.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol **74:**8358–8367. http://dx.doi.org/10.1128/JVI.74.18.8358-8367.2000.
47. **Daar ES, Li XL, Moudgil T, Ho DD.** 1990. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc Natl Acad Sci U S A **87:**6574–6578. http://dx.doi.org/10.1073/pnas.87.17.6574.
48. **Moore JP, McKeating JA, Huang YX, Ashkenazi A, Ho DD.** 1992. Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. J Virol **66:**235–243.
49. **Ringe RP, Sanders RW, Yasmeen A, Kim HJ, Lee JH, Cupo A, Korzun J, Derking R, van Montfort T, Julien J-P, Wilson IA, Klasse PJ, Ward AB, Moore JP.** 2013. Cleavage strongly influences whether soluble HIV-1 envelope glycoprotein trimers adopt a native-like conformation. Proc Natl Acad Sci U S A **110:**18256–18261. http://dx.doi.org/10.1073/pnas.1314351110.
50. **Pugach P, Ozorowski G, Cupo A, Ringe R, Yasmeen A, de Val N, Derking R, Kim HJ, Korzun J, Golabek M, de los Reyes K, Ketas TJ, Julien J-P, Burton DR, Wilson IA, Sanders RW, Klasse PJ, Ward AB, Moore JP.** 2015. A native-like SOSIP664 trimer based on an HIV-1 subtype B env gene. J Virol **89:**3380–3395. http://dx.doi.org/10.1128/JVI.03473-14.
51. **Tran K, Poulsen C, Guenaga J, de Val N, de Val Alda N, Wilson R,**

Sundling C, Li Y, Stanfield RL, Wilson IA, Ward AB, Karlsson Hedestam GB, Wyatt RT. 2014. Vaccine-elicited primate antibodies use a distinct approach to the HIV-1 primary receptor binding site informing vaccine redesign. Proc Natl Acad Sci U S A **111:**E738–E747. http://dx.doi.org/10.1073/pnas.1319512111.

52. **Khurana S, Wu J, Dimitrova M, King LR, Manischewitz J, Graham BS, Ledgerwood JE, Golding H.** 2013. DNA priming prior to inactivated influenza A(H5N1) vaccination expands the antibody epitope repertoire and increases affinity maturation in a boost-interval-dependent manner in adults. J Infect Dis **208:**413–417. http://dx.doi.org/10.1093/infdis/jit178.
53. **Ledgerwood JE, Zephir K, Hu Z, Wei C-J, Chang L, Enama ME, Hendel CS, Sitar S, Bailer RT, Koup RA, Mascola JR, Nabel GJ, Graham BS, VRC 310 Study Team.** 2013. Prime-boost interval matters: a randomized phase 1 study to identify the minimum interval necessary to observe the H5 DNA influenza vaccine priming effect. J Infect Dis **208:**418–422. http://dx.doi.org/10.1093/infdis/jit180.
54. **Strikas RA, Centers for Disease Control and Prevention (CDC), Advisory Committee on Immunization Practices (ACIP), ACIP Child/Adolescent Immunization Work Group.** 2015. Advisory committee on immunization practices recommended immunization schedules for persons aged 0 through 18 years–United States, 2015. MMWR Morb Mortal Wkly Rep **64:**93–94.
55. **Soldemo M, Pedersen GK, Hedestam GBK.** 2014. HIV-1 Env-specific memory and germinal center B cells in C57BL/6 mice. Viruses **6:**3400–3414. http://dx.doi.org/10.3390/v6093400.
56. **Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, Buckler-White A, Seaman M, Piatak M, Lifson JD, Dimitrov D, Nussenzweig MC, Martin MA.** 2013. Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. Nature **503:**277–280.
57. **Plotkin SA, Orenstein WA, Offit PA.** 2012. Vaccines, 6th ed. Elsevier, Philadelphia, PA.
58. **Zhang Y, Meyer-Hermann M, George LA, Figge MT, Khan M, Goodall M, Young SP, Reynolds A, Falciani F, Waisman A, Notley CA, Ehrenstein MR, Kosco-Vilbois M, Toellner K-M.** 2013. Germinal center B cells govern their own fate via antibody feedback. J Exp Med **210:**457–464. http://dx.doi.org/10.1084/jem.20120150.
59. **Garrity RR, Rimmelzwaan G, Minassian A, Tsai WP, Lin G, de Jong JJ, Goudsmit J, Nara PL.** 1997. Refocusing neutralizing antibody response by targeted dampening of an immunodominant epitope. J Immunol **159:**279–289.
60. **Selvarajah S, Puffer BA, Lee F-H, Zhu P, Li Y, Wyatt R, Roux KH, Doms RW, Burton DR.** 2008. Focused dampening of antibody response to the immunodominant variable loops by engineered soluble gp140. AIDS Res Hum Retrovir **24:**301–314. http://dx.doi.org/10.1089/aid.2007.0158.
61. **Yasmeen A, Ringe R, Derking R, Cupo A, Julien J-P, Burton DR, Ward AB, Wilson IA, Sanders RW, Moore JP, Klasse PJ.** 2014. Differential binding of neutralizing and non-neutralizing antibodies to native-like soluble HIV-1 Env trimers, uncleaved Env proteins, and monomeric subunits. Retrovirology **11:**41. http://dx.doi.org/10.1186/1742-4690-11-41.
62. **Harris A, Borgnia MJ, Shi D, Bartesaghi A, He H, Pejchal R, Kang YK, Depetris R, Marozsan AJ, Sanders RW, Klasse PJ, Milne JLS, Wilson IA, Olson WC, Moore JP, Subramaniam S.** 2011. Trimeric HIV-1 glycoprotein gp140 immunogens and native HIV-1 envelope glycoproteins display the same closed and open quaternary molecular architectures. Proc Natl Acad Sci U S A **108:**11440–11445. http://dx.doi.org/10.1073/pnas.1101414108.
63. **McLellan JS, Pancera M, Carrico C, Gorman J, Julien J-P, Khayat R, Louder R, Pejchal R, Sastry M, Dai K, O'Dell S, Patel N, Shahzad-ul Hussan S, Yang Y, Zhang B, Zhou T, Zhu J, Boyington JC, Chuang G-Y, Diwanji D, Georgiev I, Kwon YD, Lee D, Louder MK, Moquin S, Schmidt SD, Yang Z-Y, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Burton DR, Koff WC, Walker LM, Phogat S, Wyatt R, Orwenyo J, Wang L-X, Arthos J, Bewley CA, Mascola JR, Nabel GJ, Schief WR, Ward AB, Wilson IA, Kwong PD.** 2011. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature **480:**336–343. http://dx.doi.org/10.1038/nature10696.
64. **Kong L, Lee JH, Doores KJ, Murin CD, Julien J-P, Mcbride R, Liu Y, Marozsan A, Cupo A, Klasse PJ, Hoffenberg S, Caulfield M, King CR, Hua Y, Le KM, Khayat R, Deller MC, Clayton T, Tien H, Feizi T, Sanders RW, Paulson JC, Moore JP, Stanfield RL, Burton DR, Ward AB, Wilson IA.** 2013. Supersite of immune vulnerability on the glycosylated face of HIV-1 envelope glycoprotein gp120. Nat Struct Mol Biol **20:**796–803. http://dx.doi.org/10.1038/nsmb.2594.
65. **Zhou T, Georgiev I, Wu X, Yang Z-Y, Dai K, Finzi A, Kwon YD, Scheid JF, Shi W, Xu L, Yang Y, Zhu J, Nussenzweig MC, Sodroski J, Shapiro L, Nabel GJ, Mascola JR, Kwong PD.** 2010. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science **329:**811–817. http://dx.doi.org/10.1126/science.1192819.
66. **Blattner C, Lee JH, Sliepen K, Derking R, Falkowska E, la Peña de AT, Cupo A, Julien J-P, van Gils M, Lee PS, Peng W, Paulson JC, Poignard P, Burton DR, Moore JP, Sanders RW, Wilson IA, Ward AB.** 2014. Structural delineation of a quaternary, cleavage-dependent epitope at the gp41-gp120 interface on intact HIV-1 Env trimers. Immunity **40:**669–680. http://dx.doi.org/10.1016/j.immuni.2014.04.008.
67. **Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A, Bhattacharya T, Migueles SA, Wyatt RT, Korber BT, Mascola JR, Connors M.** 2010. Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. J Virol **84:**1631–1636. http://dx.doi.org/10.1128/JVI.01482-09.
68. **Gray ES, Taylor N, Wycuff D, Moore PL, Tomaras GD, Wibmer CK, Puren A, DeCamp A, Gilbert PB, Wood B, Montefiori DC, Binley JM, Shaw GM, Haynes BF, Mascola JR, Morris L.** 2009. Antibody specificities associated with neutralization breadth in plasma from human immunodeficiency virus type 1 subtype C-infected blood donors. J Virol **83:**8925–8937. http://dx.doi.org/10.1128/JVI.00758-09.
69. **Tomaras GD, Binley JM, Gray ES, Crooks ET, Osawa K, Moore PL, Tumba N, Tong T, Shen X, Yates NL, Decker J, Wibmer CK, Gao F, Alam SM, Easterbrook P, Abdool Karim S, Kamanga G, Crump JA, Cohen M, Shaw GM, Mascola JR, Haynes BF, Montefiori DC, Morris L.** 2011. Polyclonal B cell responses to conserved neutralization epitopes in a subset of HIV-1-infected individuals. J Virol **85:**11502–11519. http://dx.doi.org/10.1128/JVI.05363-11.
70. **Stamatatos L, Morris L, Burton DR, Mascola JR.** 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? Nat Med **15:**866–870.
71. **Wu L, Oficjalska K, Lambert M, Fennell BJ, Darmanin-Sheehan A, Ní Shúilleabháin D, Autin B, Cummins E, Tchistiakova L, Bloom L, Paulsen J, Gill D, Cunningham O, Finlay WJJ.** 2012. Fundamental characteristics of the immunoglobulin VH repertoire of chickens in comparison with those of humans, mice, and camelids. J Immunol **188:**322–333. http://dx.doi.org/10.4049/jimmunol.1102466.
72. **Popkov M, Mage RG, Alexander CB, Thundivalappil S, Barbas CF, III, Rader C.** 2003. Rabbit immune repertoires as sources for therapeutic monoclonal antibodies: the impact of kappa allotype-correlated variation in cysteine content on antibody libraries selected by phage display. J Mol Biol **325:**325–335. http://dx.doi.org/10.1016/S0022-2836(02)01232-9.
73. **Link JM, Larson JE, Schroeder HW.** 2005. Despite extensive similarity in germline DH and JH sequence, the adult Rhesus macaque CDR-H3 repertoire differs from human. Mol Immunol **42:**943–955. http://dx.doi.org/10.1016/j.molimm.2004.09.027.
74. **Wu TT, Johnson G, Kabat EA.** 1993. Length distribution of CDRH3 in antibodies. Proteins **16:**1–7. http://dx.doi.org/10.1002/prot.340160102.
75. **Yu L, Guan Y.** 2014. Immunologic basis for long HCDR3s in broadly neutralizing antibodies against HIV-1. Front Immunol **5:**250.
76. **Crooks ET, Tong T, Chakrabarti B, Narayan K, Georgiev IS, Menis S, Huang X, Kulp D, Osawa K, Muranaka J, Stewart-Jones G, DeStefano J, O'Dell S, Labranche C, Robinson JE, Montefiori DC, McKee K, Du SX, Doria-Rose N, Kwong PD, Mascola JR, Zhu P, Schief WR, Wyatt RT, Whalen RG, Binley JM.** 2015. Vaccine-elicited tier 2 HIV-1 neutralizing antibodies bind to quaternary epitopes involving glycan-deficient patches proximal to the CD4 binding site. PLoS Pathog **11:**e1004932. http://dx.doi.org/10.1371/journal.ppat.1004932.
77. **Bonhomme CJ, Knopp KA, Bederka LH, Angelini MM, Buchmeier MJ.** 2013. LCMV glycosylation modulates viral fitness and cell tropism. PLoS One **8:**e53273. http://dx.doi.org/10.1371/journal.pone.0053273.
78. **Pinschewer DD, Perez M, Jeetendra E, Bächi T, Horvath E, Hengartner H, Whitt MA, la Torre de JC, Zinkernagel RM.** 2004. Kinetics of protective antibodies are determined by the viral surface antigen. J Clin Investig **114:**988–993. http://dx.doi.org/10.1172/JCI200422374.
79. **Eschli B, Zellweger RM, Wepf A, Lang KS, Quirin K, Weber J, Zinkernagel RM, Hengartner H.** 2007. Early antibodies specific for the neutralizing epitope on the receptor binding subunit of the lymphocytic choriomeningitis virus glycoprotein fail to neutralize the virus. J Virol **81:**11650–11657. http://dx.doi.org/10.1128/JVI.00955-07.

80. **Battegay M, Moskophidis D, Waldner H, Bründler MA, Fung-Leung WP, Mak TW, Hengartner H, Zinkernagel RM.** 1993. Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. J Immunol **151:**5408–5415.
81. **Hangartner L, Zinkernagel RM, Hengartner H.** 2006. Antiviral antibody responses: the two extremes of a wide spectrum. Nat Rev Immunol **6:**231–243. http://dx.doi.org/10.1038/nri1783.
82. **Bergthaler A, Flatz L, Verschoor A, Hegazy AN, Holdener M, Fink K, Eschli B, Merkler D, Sommerstein R, Horvath E, Fernandez M, Fitsche A, Senn BM, Verbeek JS, Odermatt B, Siegrist C-A, Pinschewer DD.** 2009. Impaired antibody response causes persistence of prototypic T cell-contained virus. PLoS Biol **7:**e1000080.
83. **Dosenovic P, Soldemo M, Scholz JL, O'Dell S, Grasset EK, Pelletier N, Karlsson MCI, Mascola JR, Wyatt RT, Cancro MP, Karlsson Hedestam GB.** 2012. BLyS-mediated modulation of naive B cell subsets impacts HIV Env-induced antibody responses. J Immunol **188:**6018–6026. http://dx.doi.org/10.4049/jimmunol.1200466.
84. **Jardine JG, Ota T, Sok D, Pauthner M, Kulp DW, Kalyuzhniy O, Skog PD, Thinnes TC, Bhullar D, Briney B, Menis S, Jones M, Kubitz M, Spencer S, Adachi Y, Burton DR, Schief WR, Nemazee D.** 2015. HIV-1 vaccines. Priming a broadly neutralizing antibody response to HIV-1 using a germline-targeting immunogen. Science **349:**156–161.
85. **Dosenovic P, Boehmer von L, Escolano A, Jardine J, Freund NT, Gitlin AD, McGuire AT, Kulp DW, Oliveira T, Scharf L, Pietzsch J, Gray MD, Cupo A, van Gils MJ, Yao K-H, Liu C, Gazumyan A, Seaman MS, Bjorkman PJ, Sanders RW, Moore JP, Stamatatos L, Schief WR, Nussenzweig MC.** 2015. Immunization for HIV-1 broadly neutralizing antibodies in human Ig knockin mice. Cell **161:**1505–1515. http://dx.doi.org/10.1016/j.cell.2015.06.003.
86. **Lee E-C, Liang Q, Ali H, Bayliss L, Beasley A, Bloomfield-Gerdes T, Bonoli L, Brown R, Campbell J, Carpenter A, Chalk S, Davis A, England N, Fane-Dremucheva A, Franz B, Germaschewski V, Holmes H, Holmes S, Kirby I, Kosmac M, Legent A, Lui H, Manin A, O'Leary S, Paterson J, Sciarrillo R, Speak A, Spensberger D, Tuffery L, Waddell N, Wang W, Wells S, Wong V, Wood A, Owen MJ, Friedrich GA, Bradley A.** 2014. Complete humanization of the mouse immunoglobulin loci enables efficient therapeutic antibody discovery. Nat Biotechnol **32:**356–363. http://dx.doi.org/10.1038/nbt.2825.
87. **Murphy AJ, Macdonald LE, Stevens S, Karow M, Dore AT, Pobursky K, Huang TT, Poueymirou WT, Esau L, Meola M, Mikulka W, Krueger P, Fairhurst J, Valenzuela DM, Papadopoulos N, Yancopoulos GD.** 2014. Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice. Proc Natl Acad Sci U S A **111:**5153–5158. http://dx.doi.org/10.1073/pnas.1324022111.
88. **Macdonald LE, Karow M, Stevens S, Auerbach W, Poueymirou WT, Yasenchak J, Frendewey D, Valenzuela DM, Giallourakis CC, Alt FW, Yancopoulos GD, Murphy AJ.** 2014. Precise and in situ genetic humanization of 6 Mb of mouse immunoglobulin genes. Proc Natl Acad Sci U S A **111:**5147–5152. http://dx.doi.org/10.1073/pnas.1323896111.
89. **Schwickert TA, Victora GD, Fooksman DR, Kamphorst AO, Mugnier MR, Gitlin AD, Dustin ML, Nussenzweig MC.** 2011. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. J Exp Med **208:**1243–1252. http://dx.doi.org/10.1084/jem.20102477.
90. **Gitlin AD, Mayer CT, Oliveira TY, Shulman Z, Jones MJK, Koren A, Nussenzweig MC.** 2015. T cell help controls the speed of the cell cycle in germinal center B cells. Science **349:**643–646.
91. **Guttman M, Garcia NK, Cupo A, Matsui T, Julien J-P, Sanders RW, Wilson IA, Moore JP, Lee KK.** 2014. CD4-induced activation in a soluble HIV-1 Env trimer. Structure **22:**974–984. http://dx.doi.org/10.1016/j.str.2014.05.001.